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Evaluation of desiccation-induced oxidative injury in human red blood cells

by

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This thesis is dedicated to my parents Meir and Yardena Kanias

for their love and support throughout the years

Abstract

The current practice of red blood cell banking for transfusion medicine relies primarily on a six-week liquid storage. A growing demand for red blood cell (RBC) products has prompted the search for alternative preservation methods including dry storage. Being desiccation sensitive, attempts to recover RBCs from the dry state have failed.

This dissertation offers a new mechanistic understanding of desiccation-induced cellular injury that is correlated with the oxidative state of the hemoglobin. The general hypothesis states that RBC desiccation is accompanied with non-physiological oxidation of hemoglobin and, consequently, the release of toxic products capable of compromising cellular recovery through oxidative injury.

Data acquired for this dissertation demonstrates that water loss induces a drastic increase in the rate of hemoglobin oxidation, formation of intracellular reactive oxygen species (ROS), and hemolysis. Pharmacological treatments of the hemoglobin's oxygen binding site reveal that hemoglobin-induced cellular injury is more prominent in RBC samples that are partially dehydrated (about 3.5 to 5.5 g H_2O/g dry weight) than in samples that are relatively dry ($\leq$ 2 g H_2O/g dry weight). Furthermore, partially dehydrated RBC samples contain higher levels of oxidized lipids than more fully dried samples.

This dissertation also examined the role that glucose and glutathione play in enhancing desiccation tolerance of RBCs. Glucose treatment (5 mmol/L) significantly reduced ROS formation and hemolysis levels in partially dehydrated RBC samples (5.8 $\pm$ 0.3 g H_2O/g dry weight), but not in samples that are relatively dry (2.8 $\pm$ 0.5 g H_2O/g dry

weight). Treating RBCs with DL-buthionine-(S,R)-sulfoximine, a glutathione depleting agent, was correlated with reduced levels of desiccation-induced hemolysis.

This study suggests that desiccation-induced oxidative injury in RBCs is water dependent corresponding to earlier stages of water loss, in which cells can retain metabolic activity. Pharmacological treatments at this stage can significantly affect cell recovery as demonstrated with modifying the hemoglobin's oxygen binding site, glutathione depletion, and glucose supplementation. On the other hand, increased cytoplasmatic viscosity compromises biochemical reactions at lower residual moisture contents, and cellular injury is likely the result of physical and mechanical stress. These differences should be taken into consideration in the design of innovative approaches to RBC preservation.

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List of Abbreviations

2,3-DPG, 2,3-diphosphoglycerate

AGE, advanced glycation end products

ATP, adenosine triphosphate

BHT, butylated hydroxyl toluene

BSO, DL-buthionine-(*S,R*)-sulfoximine

CO, carbon monoxide

DCF, dichlorofluorescin

DeoxyHb, deoxygenated hemoglobin

FerrylHb, ferryl hemoglobin

GSH, reduced glutathione

GSSG, oxidized glutathione

Hb, hemoglobin

LDL, low density lipoproteins

MDA, malondialdehyde

MetHb, methemoglobin

NADPH, nicotinamide adenine dinucleotide phosphate

NO, nitric oxide

OxoferrylHb, oxoferryl hemoglobin

OxyHb, oxygenated hemoglobin

PS, phosphatidylserine

PUFAs, polyunsaturated fatty acids

RBC, red blood cell

ROS, reactive oxygen species

SOD, superoxide dismutase

SulfHb, sulfhemoglobin

TBA, thiobarbituric acid

TBARS, thiobarbituric acid reactive substances

t-BuOOH, *tert*-butyl hydroperoxide

TCA, trichloroacetic acid

CHAPTER 1: Introduction

1.1 STABILIZATION OF MAMMALIAN CELLS IN THE DRY STATE [1]

1.1.1 Drying as an alternative method for cell preservation

Would it be possible to live in a world where everybody keeps his/her own dried biological or genetic material in the pantry? This idea may seem unfathomable today, but in the last century, there have been many attempts to preserve mammalian cells in the dry state for use in regenerative medicine. Efforts to develop methods for the dry storage of human cells have been motivated by the desire for improved banking techniques, preservation of autologous stem cells, blood cells or gametes, and easier worldwide transportation of cells or other biological materials. Since the interest and need for dry storage of pharmaceuticals and foods is not a new concept, success in these industries has served to demonstrate what may be possible for mammalian cells and to encourage ongoing research in mammalian cell desiccation.

Stabilization of eukaryotic cells in the dry state is far more complicated than food substances or simple proteins. Cells have membranes, organelles such as mitochondria and nuclei, proteins, and in some cases, specific morphology that must be retained for proper functionality. In regard to this complexity, the feasibility of recovering mammalian cells from the dried state often seems questionable. Enhancing desiccation tolerance in mammalian cells requires the recognition of physiological and biochemical events that are associated with cellular injury, as well as learning and adopting survival techniques from organisms capable of surviving extreme dehydration, or desiccation.

[1] Section 1.1 is based on a review article published as Kanias, T.; Acker, J. P. Mammalian cell desiccation: Facing the challenges. Cell Preserv Technol 4:253-277; 2006.

1.1.2 Physiological effects of desiccation stress in cells

Mammalian cells lack natural mechanisms that would allow them to cope with extreme drying conditions. Water loss is correlated with changes in cell volume in response to osmotic pressure, shrinkage of cell organelles, downregulation of metabolism, increases in intracellular salt concentrations and cell viscosity, loss of enzyme activity, increased production of reactive oxygen species (ROS) and consequently, initiation of oxidative injury [1, 2].

In response to desiccation stress, the cell membrane undergoes tremendous change that may be deleterious to the cells. Rupture of the plasma membrane commonly occurs during freeze-drying of mammalian cells. The extensive loss of water causes the nonaqueous cell components such as membranes to condense, leading to morphological changes from a lipid bilayer conformation to a deleterious hexagonal II phase, which is believed to be involved in the mediation of membrane fusion [3]. Other symptoms of membrane damage are loss of membrane asymmetry, loss of membrane surface area and the release of membrane microvesicles [4, 5].

Cell organelles such as the nucleus and mitochondria are also subject to injuries during desiccation stress. Dehydration of the cell nucleus may result in chromosomal aberrations as has been observed in attempts to freeze-dry mammalian spermatozoa [6]. One of the mechanisms leading to DNA damage during desiccation is the induction of free radicals [7]. Work with isolated mitochondria from rat liver has shown that the mitochondria can become damaged at relatively high moisture levels, which suggest that protection of the mitochondria will be important in the successful desiccation of mammalian cells [8].

Desiccation can also compromise protein structure, which can result in denaturation or loss of biological activity [9]. Denaturation occurs when the covering of water on the protein surface (hydration shield) is removed, leading to disruption of the native shape of the protein as well as protein aggregation [10]. It is thought that the ability of cells to retain normal biological activity after desiccation and reconstitution without major changes to the cell proteome is unlikely. This is particularly the case for denucleated cells such as matured red blood cells (RBCs) that have no capacity to upregulate protein expression or compensate for desiccation-induced damage. Such characteristic possesses an exceptional challenge to recover RBCs from the dried state. The effects of desiccation stress on mammalian cells are summarized in Figure 1.1.

1.1.3 Anhydrobiosis and cellular protectants

Our current understanding of how to successfully cope with extreme dehydration derives primarily from studies of desiccation-tolerant organisms, or anhydrobiots. Anhydrobiosis is a diverse phenomenon found in bacteria, yeast, plants and small organisms, such as rotifers and some nematodes [11, 12]. Protection against desiccation injury is granted by various mechanisms including downregulation of metabolic activity, reduction in intracellular pH levels that is believed to hinder proteolysis, changes in membrane phosphatidylethanolamine (PE) content to minimize the formation of the hexagonal II phase, and accumulation of cellular protectants [13, 14].

Synthesis of cellular protectants requires an energy source that is highly dependent on functional mitochondria. Studies of anhydrobiots such as pea seeds have shown that upon desiccation, the mitochondria benefits from protective and repair mechanisms at the protein and membrane levels [14]. Cellular protectants are diverse and can be categorized into proteins, polyols such as glycerol, non-reducing disaccharides

such as sucrose and trehalose, polymers such as fructans, and antioxidants such as ascorbate and tocopherol [1, 15-17].

Polyols and non-reducing disaccharides have been successfully adopted for the preservation of biomaterials. RBCs, for instance, are frozen in the presence of glycerol [18], while many freeze-dried protein formulations require the use of sucrose or trehalose [10]. The role trehalose plays in protecting cells against desiccation injury is beyond the scope of this dissertation and is discussed elsewhere [2, 19-22].

1.1.4 Preservation of human RBCs

The current practice of RBC preservation relies primarily on hypothermic storage (1-6 °C) in the presence of additive solutions such as saline-adenine-glucose-mannitol (SAGM), Adsol (AS-1), Nutricel (AS-3), or Optisol (AS-5) [23]. These solutions prolong the hypothermic storage for up to 42 days. Cryopreservation of RBC units requires the use of glycerol to protect against freezing injury. The high concentrations of the added glycerol (20 % or 40 % w/v depending on the freezing protocol) are toxic; consequently, glycerol must be washed prior to transfusion to avoid the risk of intravascular hemolysis. This procedure limits the use of frozen RBC units to rare RBC phenotypes, autologous transfusion and military use [24]. A broader use of cryopreserved RBCs is not economically feasible due to complicated processing and high cost of storage [23].

The enormous need for RBCs and blood products for clinical use has driven many research groups to investigate the feasibility of RBC dry storage (for a historical review see Kanias and Acker [2]). The majority of these attempts resulted in poor cellular recovery characterized by irreversible membrane injury and cell death (hemolysis) [5, 25-31]. Cellular injury has been ascribed primarily to alterations in membrane properties in

response to osmotic stress including loss of surface area [4], microvesicle formation, and phospholipid depletion [5]. A less recognised mechanism capable of promoting cellular injury during desiccation is oxidative stress. Oxidation has been shown to compromise the longevity and recovery of desiccation-tolerant organisms including resurrection plants, lichen, plant seeds, bacteria and yeast [32, 33]. In the field of RBC preservation, this issue remains unsolved.

Human RBCs are susceptible to oxidative injury due to high concentrations of molecular oxygen, heme-bound and free iron atoms, and membrane polyunsaturated fatty acids [34]. Initiation of oxidative injury is commonly associated with changes in the red blood cell antioxidant reducing capacity, such as glutathione depletion, as well as uncontrolled autoxidation of the oxygen carrier protein, hemoglobin (Hb). Keeping the Hb in its reduced state throughout hypothermic, frozen or dry storage is a major concern because Hb oxidation releases toxic substances, which can significantly compromise cellular recovery. Section 1.2 discusses some of the putative pathways that link Hb oxidation and cellular injury during disease and during *ex vivo* preservation of RBCs.

1.2 HEMOGLOBIN OXIDATION PATHWAYS[2]

1.2.1 A very unique heme protein

One of the most fascinating members of the hemoproteins family is hemoglobin. This oxygen carrier protein is believed to have evolved from an ancestral gene over 1.8 billion years ago; preceding the divergence of prokaryotes and eukaryotes [35]. Hb genes are ubiquitous to all kingdoms of organisms including bacteria, fungi, protozoan, plants and animals [36]. Hemoglobins are vital for the metabolic function of various organisms including plants, where they can act symbiotically with microorganisms in the process of nitrogen fixation [37], or maintain cellular energy under hypoxia by acting as oxygenase in the glycolytic pathway [38].

Mammalian Hb is best known for its oxygen carrying capacity, which facilitates oxygen delivery from the lungs to respiring tissues. The human body contains approximately 750 g of Hb [39] that is mostly encapsulated in RBCs. The mature RBC contains approximately 270 million Hb molecules, which comprise more than 95 % of the cytoplasmatic proteins [40]. Each Hb molecule is capable of binding up to four oxygen molecules, therefore enabling each RBC to carry over one billion oxygen molecules. In regard to this magnificent quality, it is not difficult to imagine the consequences of Hb disorders on an individual's well-being.

There are numerous causes of hemoglobin-related pathologies and one can distinguish between those that are genetically inherited, such as thalassemias and sickle cell disease [41, 42], and those that are acquired, as in the cases of drug-induced

[2] Section 1.2 is based on a review article published as Kanias, T.; Acker, J. P. Biopreservation of red blood cells - the struggle with hemoglobin oxidation. FEBS J. 277:343-356; 2010.

methemoglobinemia [43, 44], iron deficiency anemia due to insufficient dietary iron [45], or repeated blood donations [46]. Despite the different causes, these Hb disorders share similar fates characterized by oxidative denaturation of Hb, membrane lipid and protein oxidation, hemolysis, and the release of Hb, or its denatured products, into the circulation [47-49].

The clinical consequences of excessive amounts of free Hb in the circulation have been eloquently described by Rother *et al*. [50]. Hb acts as a nitric oxide (NO) scavenger, and once released from its intrinsic environment, can significantly deplete NO plasma levels. The latter has been linked to compromised regulation of blood flow, smooth muscle dystonias, and intravascular thrombosis [50]. Extracellular Hb can rapidly oxidize to methemoglobin (metHb) through its interactions with NO [43], or when endogenous mechanisms to eliminate it, such as haptoglobin-mediated clearance, are exhausted [51]. MetHb and its denatured products, such as hemin, have been shown to intensify inflammation response of vessel endothelial cells, and to promote atherosclerosis through the oxidation of low density lipoproteins (LDL) [43].

Compared with our understanding of Hb oxidative injuries *in vivo*, little is known about the extent of Hb oxidation during *ex vivo* storage of RBCs. This issue draws interest from the fields of transfusion medicine and blood banking, as growing evidence indicates the involvement of oxidized Hb in degenerative processes pertaining to liquid storage of RBC products. Accumulation of oxidized Hb, and formation of cross-links between Hb and membrane proteins were recently demonstrated in RBCs subjected to prolonged hypothermic storage [52]. Hb oxidative injury was also evident in the attempts to develop alternative preservation methods including frozen-dried RBCs [5, 27], and Hb-based oxygen carriers for oxygen therapeutics [53].

1.2.2 Hemoglobin structure and autoxidation pathway

A major event in the evolution of vertebrate Hb occurred 450 million years ago, when gene duplication led to the creation of α-globin and β-globin genes and subsequently, to the α and β globin subunits of mammalian Hbs [36]. Human Hb is a tetramer consisting of one pair of α-like globin chains and a pair of non- α, or β-like chains. α-like chains are encoded by a cluster of genes on the short arm of chromosome 16, while β-like chains are encoded by a cluster of genes located on the short arm of chromosome 11 [54]. The most common human Hbs are the major adult Hb-A (97 %), the minor adult Hb-A_2 (2.5 %) and the fetal Hb (Hb-F; 80 % in newborns) [40, 54].

Each globin chain contains a hydrophobic pocket that is bound to a prosthetic heme group. The latter consists of an iron atom located at the center of a porphyrin ring. Through its six coordination positions, the iron atom forms four bonds with the porphyrin nitrogens, one covalent bond with a histidine residue of the polypeptide chain, and a sixth bond with exogenous ligands, such as oxygen and carbon monoxide [54]. In the absence of ligands in the sixth coordination position, the heme iron is in the high-spin ferrous state [55]. The binding of ligands involves a conformation change into a low-spin hexacoordination [55, 56]. The hydrophobic environment of the heme pocket prevents the autoxidation of the heme iron. Therefore, mutations in this region may severely compromise oxygen binding capabilities [54]. The different oxidative states of the heme iron are depictured in Figure 1.2.

Oxygen binding is accompanied with significant conformational changes to the Hb molecule. The deoxygenated structure of Hb (T state) has a low affinity to oxygen and a high affinity for heterotropic effectors including protons, chloride, carbon dioxide and organic phosphates, such as 2,3-diphosphoglycerate (2,3-DPG). Oxygenated Hb (R state),

has a higher affinity to oxygen and lower affinity to the effectors. The conformational changes are based on the ability of the heterotropic effectors to preferentially bind to deoxygenated Hb and promote the release of oxygen [54, 57].

Oxygenated Hb (oxyHb) is often denoted in the literature as $HbFe^{2+}O_2$, or $HbFe(II)O_2$ [58]. However, the binding of oxygen to Hb is accompanied with a migration of charge from the heme iron to the oxygen, such that the structure of oxyHb is $Fe^{3+}\ O_2^{\cdot-}$ [59]. OxyHb is a fairly stable molecule but does slowly autooxidize to metHb at a rate of about 0.5 % to 3 % per day [43]. The autoxidation of oxyHb involves the dissociation of the oxygen without electron transfer to yield superoxide ($O_2^{\cdot-}$) and metHb (HbFe(III)) [59].

Autoxidation of Hb

$$\mathbf{HbFe(II)O_2 \xrightarrow{autoxidation} HbFe(III)+O_2^{\cdot-}} \qquad \mathbf{(1.1)}$$

MetHb is defined as a ferric derivative of Hb, in which the sixth coordination position of the heme iron is occupied by hydroxide or water [44]. It cannot reversibly bind oxygen under physiological partial oxygen pressure [60] and is normally reduced back to deoxygenated Hb (deoxyHb) by NADH-cytochrome b5 metHb reductase [61]. The second product of Hb autoxidation, $O_2^{\cdot-}$, is a reactive oxygen species (ROS), which can be toxic if not eliminated. Normally, the RBC is equipped with extraordinary mechanisms to protect against ROS damage. Cytosolic enzymes, such as superoxide dismutase (SOD) and catalase, and the antioxidant glutathione detoxify ROS, while membrane antioxidants, such as α-tocopherol (α-Toc, vitamin E) and ascorbate (vitamin C) protect against ROS-induced lipid peroxidation [34]. Equations 1.2 to 1.5 outline some of the basic reactions between ROS and RBC antioxidants [34].

Superoxide dismutation by SOD

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1.2)$$

Elimination of the hydrogen peroxide (H_2O_2) by reduced glutathione (GSH) and glutathione peroxidase

$$H_2O_2 \xrightarrow{2GSH\ to\ GSSG} 2H_2O \qquad (1.3)$$

Reduction of oxidized glutathione (GSSG) is catalyzed by glutathione reductase

$$GSSG + NADPH + H^+ \xrightarrow{glutathione\ reductase} 2GSH + NADP^+ \quad (1.4)$$

Elimination of hydrogen peroxide by catalase

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \quad (1.5)$$

1.2.3 Uncontrolled hemoglobin oxidation and membrane damage

Various conditions can increase the rate of Hb autoxidation including cell senescence [62], hypoxia [63], and RBC pathologies such as sickle cell disease, thalassemias, glucose-6-phosphate dehydrogenase deficiency [64] and methemoglobinemia [43]. One way to evaluate Hb-induced cellular damage is by looking at the membrane, which is considered a major target for denatured Hb products and ROS attack [65]. The link between Hb oxidation and membrane lipid peroxidation has been demonstrated in the studies of hemolytic anemia [48, 66-68]. This section focuses on two of the major mediators of Hb-induced membrane injury. The culprits are hydrogen peroxide and metHb.

The role of hydrogen peroxide in promoting oxidative denaturation of Hb - H_2O_2 has unique characteristics, which distinguishes it from other ROS. Unlike superoxide that is negatively charged and, thus, requires anion channels to cross membranes, H_2O_2 is neutral and can readily cross. It has a longer half-life than other ROS and may participate

in cell signal transduction pathways through its reactions with protein thiol groups [69]. Most of the RBC intracellular H_2O_2 is generated by the dismutation of $O_2^{\cdot-}$ (Eq. 1.2), which is the product of Hb autoxidation (Eq. 1.1) [59]. Normally, reduced glutathione (Eq. 1.3) and catalase (Eq. 1.5) eliminate H_2O_2 to maintain its intracellular concentration at a steady state of approximately 0.2 nmol/L [58].

H_2O_2 is considered to be a significant mediator in the reactions between oxidized Hb and membrane lipids under oxidative stress [70]. Model studies of liposomes containing purified Hb show that addition of catalase inhibited Hb oxidation and lipid peroxidation, while addition of SOD had no effect on the oxidative damage. It was concluded that H_2O_2, but not $O_2^{\cdot-}$, mediates the oxidative damage [71]. The redox reactions between H_2O_2 and heme proteins have been summarized in a number of excellent research articles and reviews [39, 44, 53, 58, 72-77]. This section discusses some of the major pathways that may promote heme degradation during *ex vivo* storage of RBCs.

Hydrogen peroxide can react with oxyHb or metHb to yield ferrylhemoglobin (ferrylHb, HbFe(IV)=O) and oxoferrylhemoglobin (oxoferrylHb, $^{\cdot}$HbFe(IV)=O), respectively. Both products are strong oxidizing agents [58, 78].

Oxidation of oxyHb by H_2O_2

$$\mathbf{HbFe(II)O_2 + H_2O_2 \longrightarrow HbFe(IV){=}O + H_2O + O_2} \quad \mathbf{(1.6)}$$

Oxidation of metHb by H_2O_2

$$\mathbf{HbFe(III) + H_2O_2 \longrightarrow {}^{\cdot}HbFe(IV){=}O + H_2O} \quad \mathbf{(1.7)}$$

FerrylHb can further react with hydrogen peroxide to yield metHb, superoxide and water.

Oxidation of ferrylHb by H_2O_2

$$\mathbf{HbFe(IV)=O + H_2O_2 \longrightarrow HbFe(III) + O_2^{\cdot-} + H_2O} \quad \mathbf{(1.8)}$$

This reaction is believed to promote heme degradation through the production of superoxide within the hydrophobic heme pocket. SOD cannot enter the heme pocket and dismutate the superoxide that, in turn, oxidizes the tetrapyrrole rings. Moreover, the putative protonation of the superoxide in the heme pocket yields perhydroxyl radical ($^{\cdot}$OOH) that is known to initiate lipid peroxidation [58]. Superoxide formation is partially hindered by competitive reactions between oxoferrylHb and hydrogen peroxide, which generate oxygen instead of superoxide [58].

Oxidation of oxoferrylHb by H_2O_2

$$\mathbf{^{\cdot}HbFe(IV)=O + H_2O_2 \longrightarrow HbFe(III) + O_2 + H_2O} \quad \mathbf{(1.9)}$$

Summary of the reactions depicted in equations 1.7 to 1.9 indicates that Hb cycles between the ferric (Fe(III)) and the ferryl (Fe(IV)) states while it consumes hydrogen peroxide [73].

The role of metHb in promoting membrane lipid peroxidation - Several reactions can contribute to the accumulation of intracellular metHb (Equations 1.1, 1.8 and 1.9) including a putative comproportionation between ferrylHb and oxyHb, which yields two metHb molecules (Eq. 1.10) [44, 79, 80], and a direct reaction between hydrogen peroxide and deoxyHb (Eq. 1.11) [40].

Comproportionation between ferrylHb and oxyHb

$$\mathbf{HbFe(II)O_2 + HbFe(IV){=}O \longrightarrow 2HbFe(III) + O_2} \quad \mathbf{(1.10)}$$

Oxidation of deoxyHb by H_2O_2

$$\mathbf{2HbFe(II) + 2H_2O_2 \longrightarrow 2HbFe(III)H_2O + O_2} \quad \mathbf{(1.11)}$$

MetHb can react with hydrogen peroxide to generate two oxidizing species: oxoferrylHb (Eq. 1.7) and a protein radical centered on the globin chain [44, 78]. This short-lived ferryl radical reacts rapidly with oxygen to yield peroxyl radicals, which can degrade to irreversibly green Hb derivatives including choleglobin and sulfhemoglobin (sulfHb) [44, 80]. Both derivatives are similar in having covalently modified porphyrin rings and hydrophobic affinity that enables them to bind lipids. This feature has been linked to membrane damage through the extraction of membrane components, such as phosphatidylserine (PS), leading to shape change and phospholipid redistribution [80, 81].

MetHb can be converted into low-spin ferric Hb compounds named hemichromes [82]. This process is facilitated when the globin structure is destabilized or by the appearance of a suitable ligand [44]. During the conversion, the water molecule of the sixth coordination position of the metHb iron is substituted with an exogenous or endogenous ligand [83]. 'Reversible hemichromes' are commonly formed by endogenous ligands, such as globin histidine residues, and can be reduced back to deoxyHb [40]. Significant distortions in the tertiary structure of the Hb can lead to a second type of hemichromes named 'irreversible hemichromes'. The latter cannot be converted back to normal Hb [55] and are susceptible to further oxidative injury, which can result in dissociation of the polypeptide chains and release of the heme moiety [55].

The release of the heme moiety by denatured hemichromes has been shown to initiate oxidative damage to adjacent membrane components [65, 84]. The hydrophobic nature of the heme moiety facilitates its interaction with the membrane lipid domain. The rate of heme transfer into the membrane depends on several factors including the oxidation rate, the nature of the membrane lipids and the reaction medium [85, 86]. Free heme has been shown to inhibit a number of cytosolic enzymes [48], as well as to react non-specifically with membrane proteins leading to abnormal thiol oxidation and cross-linking [65].

Free heme is degraded *in vivo* through the heme oxygenase system to produce carbon monoxide, biliverdin and free iron [87]. *In vitro*, release of the heme iron has been shown to be catalyzed by intracellular GSH [65], or hydrogen peroxide [88]. Free iron is potentially toxic capable of acting as a Fenton reagent (Eq. 1.12) in the Haber-Weiss cycle, which produces the deleterious hydroxyl radicals ($^{\cdot}OH$) [86, 89].

Generation of hydroxyl radicals by heme-free iron and hydrogen peroxide

$$\mathbf{Fe(II) + H_2O_2 \longrightarrow Fe(III) + {}^{\cdot}OH + OH^-} \quad \mathbf{(1.12)}$$

Hemichrome formation has been well documented in RBC pathologies of abnormal Hbs, such as thalassemia and sickle-cell disease [48, 66-68]. Normal human Hb (HbA) is considered to be relatively resistant to hemichrome formation. However, certain conditions including heat stress, changes in pH and senescence can increase its susceptibility to hemichrome formation [82]. A summary of some of the major pathways that link Hb oxidation and membrane damage is shown in Figure 1.3.

1.2.4 Factors promoting oxidative injury in desiccated RBCs

Desiccation of RBCs can promote the autoxidation of Hb through several mechanisms including changes in pH, cell shrinkage, lower oxygen pressure and compromised antioxidant capacity. Cell shrinkage during desiccation increases the intracellular Hb concentration and can bring Hb molecules closer to the membrane surface. Hb occupies approximately 35 % of the RBC volume in the hydrated state and 95 % of the RBC dry weight [57]. A higher concentration of Hb around the cell membrane can theoretically increase the probability of cross-linking between the Hb and membrane components, such as cytoskeleton proteins and phospholipids. Thus, physical mechanisms, such as volume loss, may promote Hb-membrane interactions.

The rate of Hb autoxidation is dramatically increased under reduced oxygen pressures, reaching a maximum rate at 25 mmHg when the Hb is still partially oxygenated [90]. The increase in Hb autoxidation rate has been attributed to enhanced flexibility of the heme pocket under hypoxia, which facilitates a redox reaction between the distal histidine residue of the globin chain and the iron-bound oxygen [63, 91]. RBC desiccation is commonly performed by freeze-drying (lyophilization) or vacuum-drying under conditions simulating hypoxia (low oxygen pressures) [2], which may affect the rate of Hb oxidation.

Besides Hb, other cytosolic and membrane proteins can become compromised under desiccation stress including those participating in antioxidant defence. A recent attempt to lyophilize RBCs with high concentrations of intracellular glycerol (40 % wt/vol) [92] has demonstrated significant depletion (around 20 %) in the activity of vital enzymes (catalase, SOD and glutathione peroxidase) during 180 days of dry storage at 25 °C.

Hb oxidative injury has been prominent in recent attempts to lyophilize RBCs in the presence of trehalose [5, 27]. Introducing trehalose into mammalian cells is challenging due to the poor membrane permeability of this disaccharide [23]. One method to overcome this hurdle is by incubating RBCs at 37 °C in the presence of high concentrations of trehalose (0.8 mol/L) for about 7 h. The proposed mechanism of trehalose uptake is by osmotic imbalance and phospholipid phase transitions [93]. This method has been shown to initiate oxidative injury in terms of Hb oxidation and membrane lipid peroxidation [22].

Trehalose-loading into RBCs has been somewhat effective in retaining cellular activity after reconstitution. For instance, catalase and SOD activities have not been compromised throughout preservation, and reconstituted cells were able to synthesize ATP and their Hb secondary structure exhibited similarity to that of fresh RBCs [5]. Nevertheless, this process is still detrimental as it results in the loss of membrane phospholipids, significant levels of metHb (50 %), and hemolysis (45 %) [5]. The addition of ascorbic acid to the rehydration buffer has somewhat reversed the oxidative injury by decreasing metHb levels to about 15 %. However, these levels are still above the physiological range (0.5 to 3 %). Dry storage of trehalose-loaded RBCs at 4 °C was not resistant to Hb oxidation, as metHb levels continued to rise over a studied period of 10 weeks [27]. Selected findings of the RBC oxidative injury during desiccation stress are illustrated in Figure 1.4.

1.3 HYPOTHESIS

Desiccation stress in RBCs is accompanied by non-physiological oxidation of Hb and, consequently, the release of toxic products capable of compromising cell integrity through unrestricted oxidation of vital cellular components including the plasma membrane.

1.4 RESEARCH AIMS

Testing the hypothesis requires the demonstration that Hb oxidation is directly involved with cellular injury during desiccation stress. In order to do so, research aims are: 1.To establish methods for monitoring oxidative injury in vacuum-dried RBC specimens. 2. To demonstrate that RBC desiccation induces Hb oxidation and ROS formation, and to investigate the correlation between Hb oxidation and hemolysis. 3. To demonstrate that inhibition of Hb redox reactions during desiccation significantly affect cellular recovery post reconstitution. This dissertation also examines the effects of two stress factors, glucose starvation and glutathione depletion, on the recovery of vacuum-dried RBCs.

1.5 EXPERIMENTAL DESIGN

The majority of assays for determining oxidative stress have not been validated for use with reconstituted (i.e. vacuum-dried and rehydrated) RBCs. Moreover, numerous commercial assays are incompatible with RBCs due to Hb interference. The first research goal is, therefore, aimed to adopt and modify assays that can reliably illustrate changes in the oxidative state of reconstituted RBCs. Methods implemented in this study include spectrophotometric analysis of the Hb oxidative state (oxyHb, metHb, hemichromes), and

flow cytometric analysis of intracellular ROS and heme degradation products. Chapter 2 discusses in detail the modification of a spectrophotometric method for determining membrane lipid peroxidation in desiccated RBCs.

The second research goal is to demonstrate that desiccation induces Hb oxidative injury in RBCs and investigate its correlation to hemolysis. This goal was achieved by monitoring changes in the RBC oxidative state and hemolysis levels at defined residual moisture contents. Data shown in Chapter 3 confirms that desiccation induces oxidative injury in terms of Hb oxidation and ROS formation, and discusses the correlation between Hb oxidation and hemolysis.

In order to link Hb oxidation to cellular injury in desiccated RBCs, it was necessary to modify the Hb oxidative state prior to desiccation in a manner that would minimize redox reactions during desiccation, dry storage and rehydration. Modulations of the Hb oxidative state were achieved by treating RBCs with carbon monoxide (CO) to block the heme iron and, consequently, reduce metHb and superoxide formation during desiccation. MetHb toxicity was also investigated by vacuum-drying RBCs containing over 90 % metHb. Hb oxidation to metHb was performed prior to desiccation using sodium nitrite. These experiments clarified the role Hb plays in mediating cellular damage during RBC desiccation. Experimental data and conclusions are presented in Chapter 4.

Chapter 5 expands our understanding regarding stress factors capable of affecting the rates of oxidative injury and hemolysis in vacuum-dried RBCs. It demonstrates the conditions, by which glucose supplementation can mitigate ROS formation and hemolysis in vacuum-dried RBCs. It also examines the consequences of glutathione

depletion on the recovery of vacuum-dried RBCs, and suggests that glutathione can act as a prooxidant during desiccation stress.

Chapter 6 summarizes the results and offers a new mechanistic understanding of desiccation-induced oxidative injury in RBCs. This chapter also discusses the hypothesis and relevance of this dissertation to the fields of cell preservation and cryobiology.

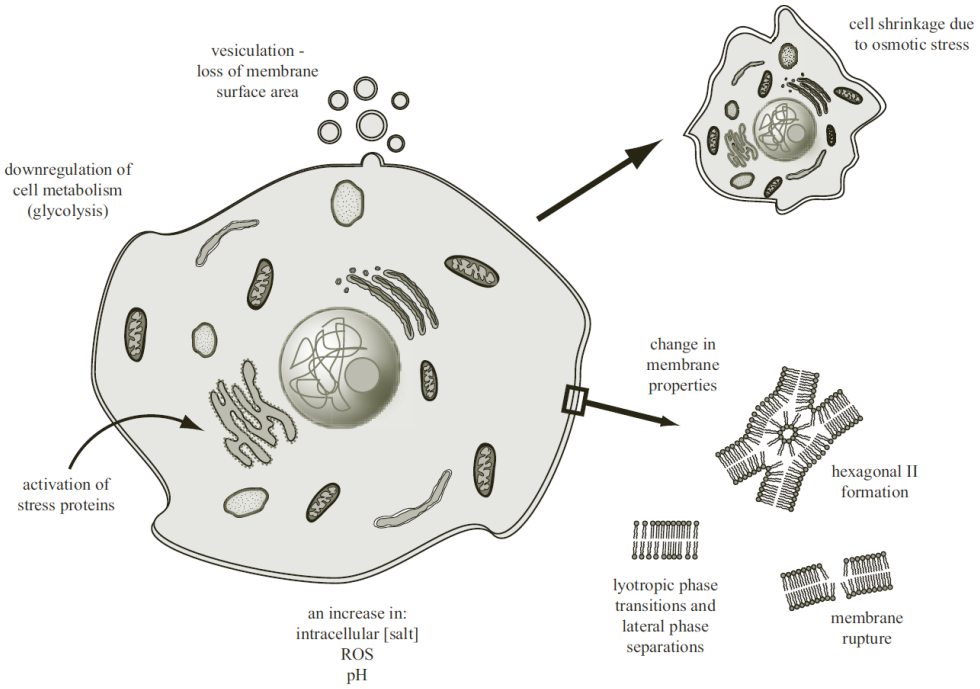

Figure 1.1. The effects of desiccation on mammalian cells. Water depletion within the cells is associated with deleterious effects, including shrinkage of cell organelles, membrane rupture, membrane vesiculation, and increased solute concentration (Adopted from Kanias and Acker, Cell Preserv Technol 4:253-277; 2006).

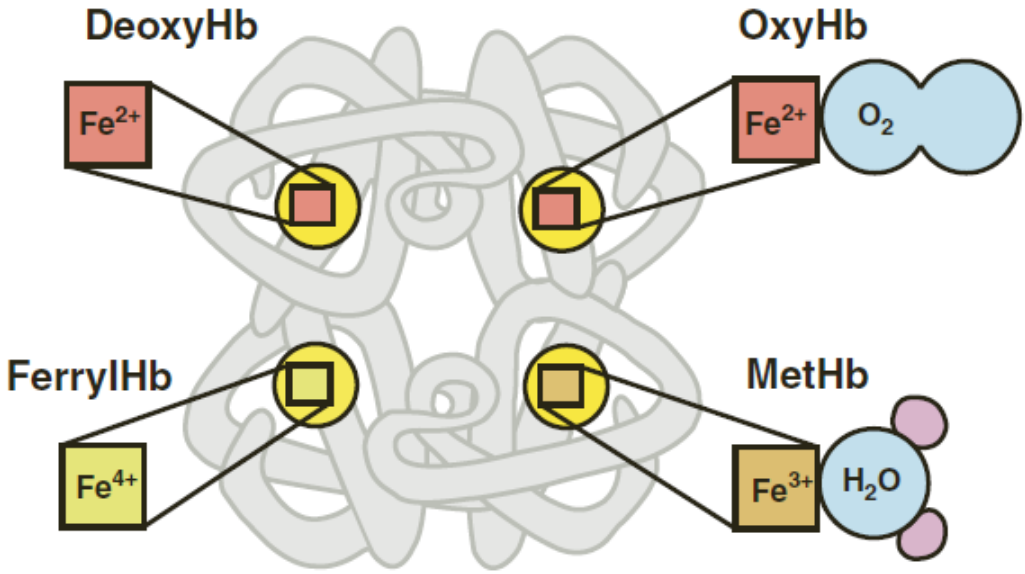

Figure 1.2. Different oxidative states of the heme iron. Each of the four globin chains of the hemoglobin (Hb) molecule contains a heme group with an iron atom at its centre. Hb oxidation can involve changes in the iron's oxidative state, and, consequently, modifications in ligand binding. The reduced ferrous state (Fe^{2+}, red) allows oxygen binding to deoxygenated Hb (deoxyHb) at physiological conditions. The resultant oxygenated Hb (oxyHb) can undergo oxidation to methemoglobin (metHb), in which the heme iron is in the ferric state (Fe^{3+}, brown). MetHb can bind water, but not oxygen under physiological conditions. The ferryl state (Fe^{4+}, green) can be obtained during Hb oxidative injury by hydrogen peroxide. This state can lead to Hb denaturation (Adopted from Kanias and Acker, Biopreservation of red blood cells - the struggle with hemoglobin oxidation. FEBS J. 277:343-356; 2010).

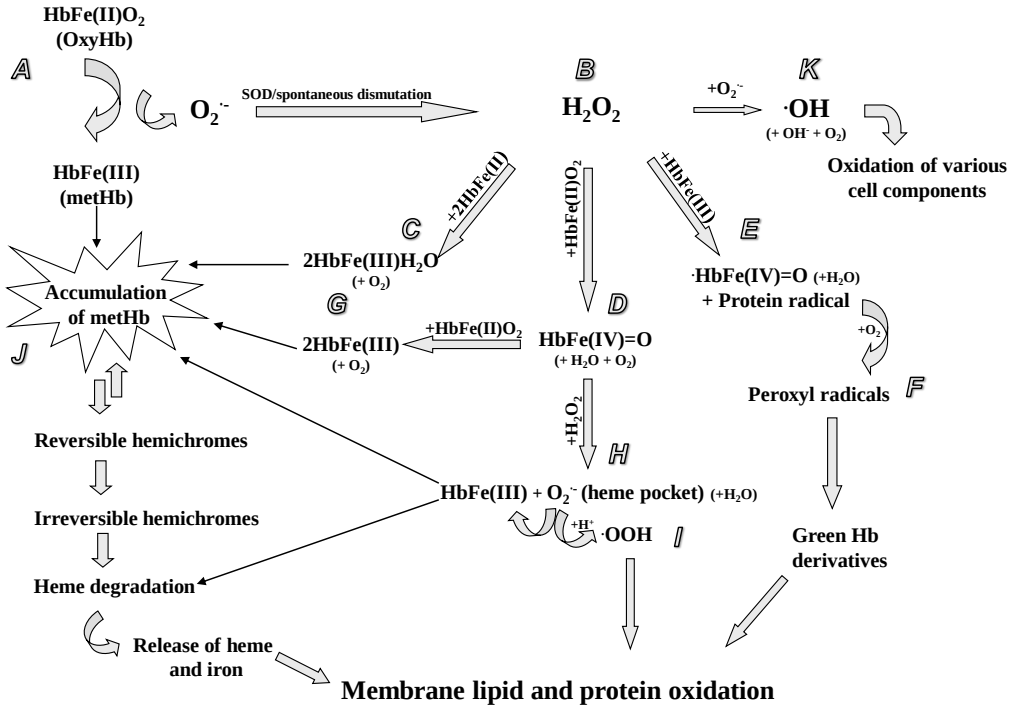

Figure 1.3. Summary of the major hemoglobin (Hb) oxidative pathways and their link to membrane damage. (A) The autoxidation of oxygenated Hb (oxyHb, $HbFe(II)O_2$) generates methemoglobin (metHb, HbFe(III)) and superoxide anions ($O_2^{\cdot-}$) [59]. Spontaneous or enzymatic dismutation of $O_2^{\cdot-}$ generates hydrogen peroxide (H_2O_2) [34]. (B) Accumulation of H_2O_2 due to the loss of vital antioxidants (catalase, glutathione) may cause irreversible oxidative damage to Hb molecules. H_2O_2 can react with deoxygenated Hb (HbFe(II)), oxyHb, and metHb to generate metHb (C), ferrylHb (HbFe(IV)=O) (D) and oxoferrylHb ($^{\cdot}$HbFe(IV)=O) (E), respectively [40, 58, 78]. The reaction between H_2O_2 and metHb (E) also generates a protein radical that can further react with oxygen to yield peroxyl radicals (F) and, eventually, decompose to lipophilic green Hb derivatives, which bind to membrane lipids and induce oxidative damage [44, 78]. FerrylHb (D) can react with oxyHb to generate two molecules of metHb (G) [44, 79, 80], or with H_2O_2 (H) to generate metHb and $O_2^{\cdot-}$ [58]. The formation of $O_2^{\cdot-}$ within the heme pocket of the Hb has been shown to result in heme degradation. Alternatively, a protonation of $O_2^{\cdot-}$ generates perhydroxyl radicals ($^{\cdot}$OOH) (I) that are known to initiate membrane lipid peroxidation [58]. Accumulation of intracellular metHb (J) may result in the formation of hemichromes. Irreversible hemichromes undergo further denaturation leading to heme degradation and release of the heme moiety and iron atoms [55]. Both degradation products have been shown to initiate membrane oxidative damage. The presence of $O_2^{\cdot-}$ and H_2O_2 may result in the formation of hydroxyl radicals ($^{\cdot}$OH) (K) through the Haber-Weiss reaction. Heme degradation releases iron atoms, which can catalyse this reaction [86, 89]. $^{\cdot}$OH is highly reactive and can cause oxidative damage to various cell components. Note that all three major oxygen radicals ($O_2^{\cdot-}$, H_2O_2 and $^{\cdot}$OH) can take place in the oxidative denaturation of Hb and membrane damage. Remark: In order to simplify this model, reaction products that do not participate in oxidative pathways (i.e. H_2O, O_2) are denoted by smaller fonts in parentheses (Adopted from Kanias and Acker, Biopreservation of red blood cells - the struggle with hemoglobin oxidation. FEBS J. 277:343-356; 2010).

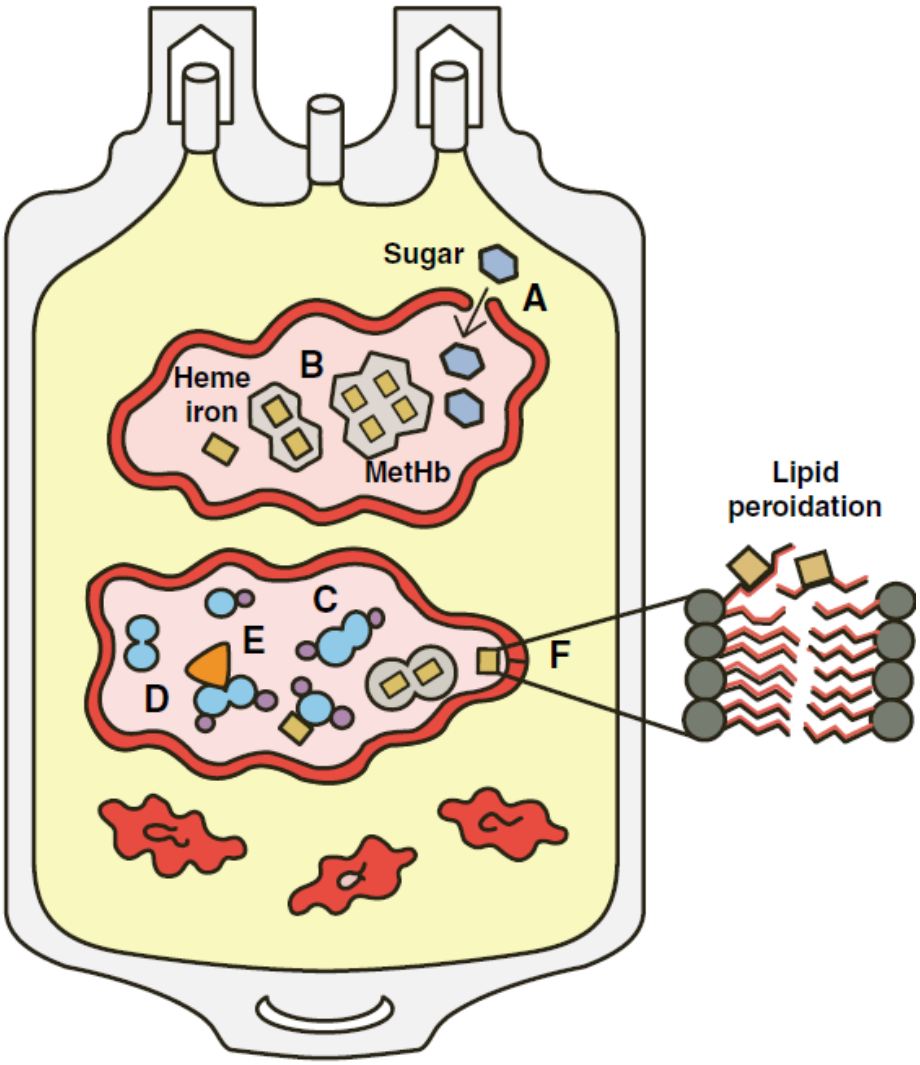

Figure 1.4. Selected findings of RBC oxidative injury during desiccation stress and dry storage. Water loss involves physiological changes, such as cell shrinkage, which can significantly increase the RBC susceptibility to oxidative injury. A. Initiation of oxidative injury may occur during the loading of intracellular protectants, such as trehalose and glucose [22, 93]. B. Abnormal levels of metHb (50 %) found in reconstituted RBCs [5] emphasize the severity of the injury. C. Hb autoxidation increases intracellular levels of ROS. D. Rehydration reintroduces oxygen, which can participate in redox reactions and exacerbate the oxidative injury. E. Lyophilization and dry storage have been associated with compromised antioxidant activity of vital enzymes (SOD, catalase, glutathione peroxidase) [92]. F. The presence oxidized Hb and ROS, as well as insufficient antioxidant defense can result in membrane damage by protein and lipid oxidation and consequently, hemolysis (Adopted from Kanias and Acker, Biopreservation of red blood cells - the struggle with hemoglobin oxidation. FEBS J. 277:343-356; 2010).

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CHAPTER 2: Determination of Lipid Peroxidation in Desiccated RBCs[3]

2.1 INTRODUCTION

Lipid peroxidation of biological membranes is one of the most studied indicators of oxidative stress [1]. The process initiates when ROS, such as hydroxyl radicals ($^{\cdot}OH$), remove a hydrogen atom from methylene carbons of fatty acid side chains [2]. Phospholipids consisted of polyunsaturated fatty acids (PUFAs) are particularly susceptible to peroxidation due to a large number of double bonds found in the latter [3]. The resulting lipid radical can react with molecular oxygen to yield peroxyl radicals. Peroxyl radicals are capable of propagating the oxidative process by removing hydrogen atoms from adjacent fatty acids, and consequentially create various lipid hydroperoxides [1]. Lipid hydroperoxides can further decompose to secondary lipid peroxidation products such as malondialdehydes (MDAs). This process is catalyzed by the presence of transition metals, such as iron [3].

MDAs have been widely used as lipid peroxidation indicators. These three-carbon aldehydes are capable of creating adducts with various cellular components including proteins, phospholipids, DNA and RNA [4, 5]. MDAs have been shown to have adverse effects on cell integrity as shown by membrane damage and hemolysis in RBCs [4]. The detection of MDAs in biological specimens is based on the reaction between MDAs with thiobarbituric acid (TBA), which yields the thiobarbituric acid reactive substances (TBARS) chromophore. The latter can be quantified spectrophotometrically at

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532 nm [3]. This method was introduced over six decades ago by Kohn and Liversedge [6].

The TBARS test is still the most commonly used assay for the measurement of lipid peroxidation in biomaterials. It is relatively simple, fast and cost-effective. On the other hand, this assay suffers from several disadvantages and has been criticized for its lack of consistency and specificity [7]. First, the large number of variations and modifications of this assay worldwide have created significant differences in the measured levels of MDAs of similar specimens. Therefore, it is often impossible to compare the results of different research groups [5]. Second, the measured values of MDAs can be affected by interfering substances (aldehydes, bile pigments, cyclic peroxides, carbohydrates, hemoglobin, glycoproteins), which contribute to the absorbance at 532 nm and thereby create false-positive results. Third, sample autoxidation during testing can also increase the obtained MDA levels [8].

Several improvements to the MDA assay have been introduced throughout the years including the addition of the antioxidant butylated hydroxyl toluene (BHT) to decrease sample autoxidation [9, 10], and the development of more specific high performance liquid chromatography (HPLC) methods [1, 11, 12]. Nevertheless, the TBARS colorimetric assay is still extensively used and can be effective for rapid screening of multiple specimens, evaluation of antioxidant efficacy, and determination of the extent of oxidative damage in biomaterials.

The measurement of TBARS in RBC specimens is especially problematic, due to Hb interference and the presence of iron, which may catalyze sample oxidation [13]. Therefore, the majority of the commercially available TBARS assay kits are not suitable

for RBCs. A method to determine MDAs in RBC specimens was introduced in the early seventies by Stocks and Dormandy [14]. The method is based on the extraction of MDAs from RBC specimens by trichloroacetic acid (TCA), followed by a reaction with TBA and measurement of the colored product at 532 nm. The MDA concentration (nmol/L) is calculated from the molar extinction coefficient determined by Yu and Sinnhuber (1.56 x 10^5 $M^{-1} \cdot cm^{-1}$) [15, 16]. Several modifications of this method were later utilized for the measurement of MDAs in human RBCs. A recent evaluation of the different methods stated that Stocks and Dormandy's method has the highest sensitivity and excellent reproducibility for determining MDAs in human RBCs [17].

RBC susceptibility to oxidative injury including redox reactions capable of initiating membrane lipid peroxidation have been discussed in detail in section 1.2 of Chapter 1. The objective of this chapter is to modify the TBARS assay for use with reconstituted RBC specimens (either vacuum-dried or lyophilized). Such an assay would be used later as a tool to understanding the correlation between Hb oxidation and membrane lipid peroxidation during desiccation, dry storage and rehydration of RBCs.

2.2 MATERIAL AND METHODS

Reagents - 2-thiobarbituric acid (TBA) was obtained from MP Biomedicals Inc (Solon, OH, USA), sodium hydroxide 0.500 mol/L from Ricca Chemical Company (Arlington, TX, USA), 2,6-di-tert-butyl-p-cresol (BHT) from Spectrum chemical Mfg Corp (Gardena, CA, USA), 1x phosphate buffered saline (PBS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L phosphate buffer, pH 7.4) was diluted from a 10x PBS stock obtained from OmniPur (Gibbstown, NJ, USA), malonaldehyde bis-(dimethyl acetal) from ACRŌS Organics (Geel, Belgium), sodium meta-arsenite from J.T. Baker (Phillipsburg, NJ, USA). 1,1,3,3-tetraethoxy-propane, acetonitrile, trichloroacetic acid,

tert-Butyl hydroperoxide solution (t-BuOOH), and Drabkin's reagent components (Triton X-100, potassium dihydrogen phosphate, potassium cyanide and potassium ferricyanide (III)) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Preparation of human red blood cell suspensions - Leukocyte-reduced packed RBC units stored in saline-adenine-glucose-mannitol (SAGM) were provided by Canadian Blood Services. These units were produced from whole blood collections obtained from healthy donors. The units' age varied from 3 to 21 days. The RBCs were washed twice with PBS (5 min, 3200x g, 4 °C), the supernatant was discarded and the RBC pellet was suspended in PBS to a final hematocrit (HCT) of about 3 %. The HCT was determined by micro-hematocrit centrifugation (Hettich Haematokrit).

Evaluation of the antioxidant BHT in reducing sample autoxidation - The efficacy of BHT in reducing sample autoxidation was tested by adding BHT stock solution (0.5 mol/L in acetonitrile) to fresh and lyophilized RBC suspensions before the assay reagents were added. The BHT final concentration in the RBC suspensions was 5 mmol/L.

Oxidation of RBCs - Oxidized RBCs were used as a positive control during the modification of this assay. RBC suspensions were washed once (5 min, 3200x g, 4 °C) and the pellet was suspended in PBS containing t-BuOOH (2 mmol/L). The RBC samples were shaken gently on a nutating mixer while incubated for 30 min at 37 °C. At the end of the incubation, the samples were washed (same conditions) with PBS to remove residual t-BuOOH, and resuspended in PBS prior to assay. Washing is essential because t-BuOOH residues were found to interfere with the assay, as seen by absorbance peaks at 453 nm and 532 nm.

Lipid peroxidation assay - MDA standard curve: MDA standards were generated from a malonaldehyde bis-(dimethyl acetal) stock. A 1 molar MDA stock was prepared by dissolving 166 µL malonaldehyde bis-(dimethyl acetal) in 834 µL ethanol. The MDA stock was further diluted with double-distilled water to produce MDA standards raging from 0.156 µmol/L to 5 µmol/L. MDA standards were also be prepared by hydrolysis of 1,1,3,3-tetraethoxy-propane (TEP) [11]. However, these standards were found to be less stable during lyophilization than those generated from malonaldehyde bis-(dimethyl acetal) (data not shown). Therefore, the use of malonaldehyde bis-(dimethyl acetal) was preferred over TEP.

MDA extraction from fresh/ lyophilized RBC samples and MDA standards: RBC samples were mixed with TCA (28 % w/v) in a 2:1 ratio. The samples were vortexed, incubated for 10 min at 20 °C, centrifuged to remove the acid precipitate (16,100x g, 10 min, 18 °C) and the supernatants (MDA extracts) were collected carefully to avoid contamination with cell debris (denatured Hb, iron). The MDA extracts were mixed in a 4:1 ratio with either TBA (1 %, dissolved in 0.05 mol/L NaOH), or 0.05 mol/L NaOH alone. The latter served as a sample blank.

The MDA standards were treated exactly like samples with the exception that no sample blanks were used. RBC extracts, sample blanks and MDA standards were placed in boil-proof microtubes with screw-top caps to avoid evaporation, incubated in boiling water for 15 min and immediately cooled on ice for 10 min. Triplicates (200 µL) from each standard and sample were transferred into a 96-well plate. The TBARS were quantified spectrophotometrically (Spectramax PLUS 384, Molecular devices, CA, USA) at 453 nm and 532 nm. Spectrophotometer optics were verified by a validation plate (SpectraTest™, Molecular devices, CA, USA).

Calculation of TBARS: RBC TBARS concentrations were determined from the MDA standard curve after correcting for errors caused by interfering substances at 453 nm using the method of Gilbert *et al*. [18].

Calculation of TBARS

TBARS (µmol/L) = [(Sample absorbance at 532 nm – sample blank absorbance at 532 nm) - 0.2 (Sample absorbance at 453 nm – sample blank absorbance at 453 nm) -*Y* intercept] /slope **(2.1)**

The final TBARS values in RBC samples are presented as nmol/ g Hb. Hemoglobin concentration (g/L) was determined by a colorimetric method using Drabkin's reagent. This method converts total hemoglobin into cyanmethemoglobin, a derivative that can be quantified spectrophotometrically at 540 nm [19].

Desiccation of RBC samples- Desiccation was obtained by freeze-drying (lyophilization). RBC specimens (4 mL) were transferred into 60x15 mm Petri-dishes, which were placed in a -80 °C freezer for 1 h. The frozen samples were then placed in a lyophilizer (Advantage 2.0 EL, VirTis, USA) and dried for 22 h (Primary drying: 16 h at -25 ºC; secondary drying: 6 h at 25 ºC; pressure ≤ 1 Torr; condenser at -85 ºC). Under these drying conditions, the residual moisture was less than 3 %. Residual moisture was determined using the gravimetric method [20]. The desiccated samples were rehydrated back to their original volumes by the addition of double-distilled water and assayed immediately for TBARS to avoid further oxidation. In part of that study, lyophilized samples in Petri dishes were covered (Petri dish's glass caps) and stored at 20 °C for 24 h or 72 h before rehydration.

Recovery of MDA standards in fresh and lyophilized RBCs - The ability to measure TBARS in desiccated RBCs was verified by the recovery of known amounts of MDAs (0.3125, 0.625, 1.25 and 5 µmol/L) spiked into fresh RBC suspensions that were either lyophilized, or assayed immediately (control). The recovery (measured absorbance) was compared to a MDA standard curve (expected absorbance).

Statistical analysis - A nonparametric statistical analysis was performed using commercial software (SPSS, Lead Technologies, Charlotte, NC, USA). The differences between two independent groups were analysed using Mann-Whitney U test, while Kruskal-Wallis ANOVA was used to examine the differences among multiple groups. Results are shown as the mean ± SD. Probabilities less than 0.05 were considered significant.

2.3 RESULTS

MDA standard curve - MDA standards produced from malonaldehyde bis-(dimethyl acetal) generate a linear standard curve (Figure 2.1) under strong acidic conditions (pH 0.7). A low pH is essential for the stability of the MDA-TBA adduct, which dissociates slowly at neutral pH [11, 21]. For this reason, the standards were treated with TCA in the same manner as the samples. The absorbance values of the MDA standards at 532 nm are similar to those obtained by Srour *et al*. for Stocks and Dormandy's method [17]. The linear regression line of the MDA standard curve is $y = 0.098x + 0.0022$, $R^2 = 0.9999$.

Recovery of MDA standards in fresh and lyophilized RBCs - The recovery of MDA standards that were spiked into fresh RBC suspensions was 94 ± 4 % over all concentrations (Figure 2.2) and is higher than the values obtained by Srour *et al*. (50 % to

85 %) [17]. The recovery of MDA standards spiked into fresh RBC suspensions, which were then lyophilized, was 73 ± 9 % over all concentrations (Figure 2.3). The lower recovery is expected, as sample loss is often associated with drying and rehydration procedures. The absorption spectra of a pure MDA standard (0.625 µmol/L), MDA standard (1.25 µmol/L) spiked into RBCs then lyophilized, and lyophilized RBCs (no MDA added) are depicted in Figure 2.4.

Determination of TBARS in fresh and lyophilized RBCs - The ability to monitor changes in TBARS concentrations of desiccated RBC samples was verified by a set of lyophilization and dry storage experiments (n = 4). RBC samples (average HCT of 3 %) were lyophilized and either rehydrated immediately at the completion of the lyophilization process, or stored at 20 °C for 24 h and 72 h prior to rehydration. Dry storage of RBC specimens under these conditions (not oxygen proof) is expected to increase the oxidative stress. Control samples consisted of fresh RBC suspensions, which were kept at 4 °C for up to 72 h. The TBARS concentrations of lyophilized RBC samples were significantly higher ($p=0.021$ at 0 h and 72 h, $p=0.019$ at 24 h) than that of fresh RBC samples at all tested times (Figure 2.5A). Dry storage of lyophilized RBCs for 24 h and 72 h resulted in a significant increase ($p= 0.019$) in TBARS concentration compared to lyophilized RBCs, which were rehydrated immediately (43 ± 6 nmol/g Hb and 46 ± 4 nmol/g Hb versus 12 ± 2 nmol/g Hb, respectively) (Figure 2.5B).

Evaluation of the antioxidant BHT in reducing sample autoxidation during the TBARS assay - The efficacy of BHT in reducing sample autoxidation during the TBARS assay was investigated in fresh RBCs, lyophilized RBCs, and lyophilized RBCs kept in dry storage for 72 h. Control samples were not treated with BHT. BHT (5 mmol/L, final concentration) significantly reduced the measured TBARS concentration in fresh RBCs

(p=0.043) and RBCs subjected to 72 h dry storage (p=0.05). However, there were no significant differences (p=0.796) between control and BHT-treated samples of lyophilized RBCs (Table 2.1).

Investigation of the interference caused by the 453 nm peak in the TBARS assay - Examination of the spectrophotometric results reveals that in some samples, the magnitude of the 453 nm peak can interfere with the 532 nm peak. The origin of the 453 nm peak in RBC specimens is not clear and is thought to be related to the presence of cell debris [22] and glutathione [18]. The magnitude of the 453 nm peak was investigated in TBARS samples from fresh RBCs, lyophilized RBCs, fresh RBCs treated with t-BuOOH, and in t-BuOOH solution (2 mmol/L in PBS), which was treated like a sample. The 453 nm peak of t-BuOOH-treated RBCs was significantly higher ($p<0.0001$) than that of fresh and lyophilized RBCs, which received no t-BuOOH treatment (Figure 2.6). The 453 nm peak of lyophilized RBCs was two times higher than that of fresh RBCs. The 453 nm peak of the t-BuOOH solution (RBC free) was similar to the one observed in t-BuOOH-treated RBCs. Measurement of the 532 nm peak of the t-BuOOH solution revealed a TBARS artefact that was equivalent to a concentration of about 17 nmol/L.

2.4 DISCUSSION

Lipid peroxidation of membrane lipids and, particularly, PUFAs has been shown to have adverse effects on cell survival through the production of deleterious intermediates including peroxyl radicals, lipid hydroperoxides and MDAs [4]. MDAs are of particular interest due to their high reactivity with various biological macromolecules including DNA, RNA and proteins [5, 11]. The presence of MDAs in biomaterials and foods is frequently used as an indicator of oxidative damage. The most common method

for quantifying MDAs is based on the spectrophotometric measurement of the chromophore generated from the reaction of MDAs, or other aldehydes, with TBA [11].

The extent of oxidative damage that occurs during the desiccation of mammalian cells is not well established. The majority of assays for determining oxidative stress have not been validated for dried mammalian specimens, mainly because most oxidative stress indicators are labile and difficult to detect in the dry state. Hence, it was necessary to verify whether MDAs that are formed during RBC desiccation stress can be monitored post sample reconstitution.

The MDA standard curve was found to be linear at the range of 0.156 µmol/L to 5 µmol/L (Figure 2.1). TBARS concentrations in fresh RBC specimens (control) were below detection or very low. This is in agreement with the literature, since freshly drawn RBCs rarely contain detectable levels of MDAs [3]. However, lipid peroxidation has been shown to evolve during hypothermic storage of RBC units [23], and may explain the low concentrations of TBARS obtained in some of the control samples.

The molar extinction coefficient of MDA was obtained from the slope of the MDA standard curve and determined to be 0.98 $x10^5$ $M^{-1}\cdot cm^{-1}$ at 532 nm. This value is lower than the one determined by Yu and Sinnhuber (1.56 $x10^5$ $M^{-1}\cdot cm^{-1}$) [15, 16]. Many research groups have based their calculation of MDAs on the molar extinction coefficient as determined by Yu and Sinnhuber without generating their own standard curve. This practice contributes to errors, since it ignores the differences in instruments and reagents between labs. Yu and Sinnhuber's coefficient was determined more than six decades ago using different procedures and different reagents (hydrochloric acid in place of TCA) [15]. Moreover, modern spectrophotometric instruments are more sensitive and accurate

than those of the 1950s. In order to improve accuracy, it is highly recommended that users of this assay generate their own MDA standard curve and that the MDA standards are treated in the same manner as samples.

Sodium arsenite is commonly added with TCA for the extraction of MDAs from RBC specimens. The addition of sodium arsenite (0.1 mol/L) was explained by its ability to stabilize the MDA chromogen so that subsequent stages of the TBARS assay may be delayed [14]. In this study, the presence of sodium arsenite in the TCA solution was found to create a strong background (absorbance of 0.034) at 532 nm after reaction with TBA. This interference may create false positive results, especially when using Yu and Sinnhuber's MDA extinction coefficient (1.56×10^5 $M^{-1} \cdot cm^{-1}$) without generating a MDA standard curve. If samples are assayed immediately for TBARS, it is recommended that sodium arsenite be omitted from the procedure. This has the added benefit of reducing the potential hazards of the TBARS assay since sodium arsenite is extremely toxic. However, if sodium arsenite must be used, a sample blank of TCA-sodium arsenite is recommended.

MDA standards were successfully recovered (94 % ± 4 %, Figure 2.2) after being spiked into fresh RBCs. There were no significant differences ($p>0.05$) between the recovery values of the tested concentrations (0.3125 µmol/L, 0.625 µmol/L, 1.25 µmol/L and 5 µmol/L). A lower recovery was obtained when the standards were lyophilized in RBC suspensions (73 % ± 9 %, Figure 2.3). This decrease in recovery can be explained by the drying procedure and cell concentration. The selected drying conditions (see material and methods) were aimed to test the recovery of MDA standards under harsh desiccation stress, characterized by rapid drying to a low residual moisture content (< 3 %) followed by rapid rehydration. The recovery of MDA standards may change under

different drying conditions. Poor recovery of MDA standards can also be the consequence of interference by cell debris. This was proven by testing the recovery of MDA standards which were lyophilized in PBS (cell free; data not shown). In this case, the recovery increased to 89 % ± 4 %.

This assay has been modified for monitoring TBARS in lyophilized RBC specimens. Since little is known about the extent of oxidative damage that occurs during the desiccation of mammalian cells, it was not initially known whether TBARS would be formed as a result of drying. However, lyophilized RBCs were shown to contain significantly higher concentrations ($p \leq 0.021$) of TBARS than fresh RBCs (Figure 2.5A). The presence of TBARS in lyophilized RBCs is not only meaningful for assay validation, but also provides first evidence for lipid peroxidation and hence, oxidative damage that is associated with desiccation. The efficacy of this assay to monitor TBARS was also verified during dry storage of lyophilized RBCs (Figure 2.5B), where significant changes in TBARS concentrations were observed after 24 h and 72 h. The ability to detect changes in TBARS concentrations during dry storage of mammalian cells will be useful when evaluating different storage conditions, or when screening for potential antioxidants.

Sample autoxidation during the TBARS assay may result in false positive errors [9]. The antioxidant BHT is commonly added to samples in order to protect lipids from subsequent oxidation [24, 25]. Elimination of sample autoxidation by BHT was introduced by Pikul *et al*. [9, 10] for the measurement of MDAs in chicken fat. The efficacy of BHT in reducing sample autoxidation of fresh and lyophilized RBC samples was found to be inconsistent (Table 2.1). This discrepancy is most likely the consequence of poor solubility of BHT in aqueous solutions. Hydrophobic BHT is soluble in organic

solvents such as acetonitrile, but will readily crystallize when diluted into aqueous solutions (RBC suspensions, TCA) at room temperature [9]. When extracting RBC lipids with TCA, BHT crystals can be trapped and lost in the cell pellet. In this case, the supernatant will contain inconsistent concentrations of BHT. The use of BHT in aqueous solutions is therefore problematic. A water-soluble antioxidant might be more efficient in protecting RBCs and other (non adipose) mammalian cells from autoxidation during the assay. However, some antioxidants may scavenge MDAs and, therefore, interfere with the assay.

The absorption spectrum of TBARS extracted from RBC specimens shows peaks at 532 nm and 453 nm (Figure 2.4). The source of the 453 nm peak in RBCs has been attributed to cell debris and glutathione [22]. The magnitude of the 453 nm peak can interfere with the 532 nm peak and contribute to false positive errors. Gilbert *et al*. developed a method to correct this error by subtracting 20 percent of the 453 nm absorbance from the 532 nm peak. Use of this correction in the TBARS assay was demonstrated by the authors to give MDA concentrations that correlate well with values obtained by HPLC [18].

Investigation of the 453 nm peak has shown that its magnitude is significantly higher in samples subjected to oxidative stress (i.e. lyophilized RBCs and t-BuOOH-treated RBCs) (Figure 2.6). Interestingly, the addition of t-BuOOH solution (2 mmol/L) to TBA produced a yellow pigment with an absorbance peak at 453 nm. This suggests that hydroperoxides are, at least partially, the source of the 453 nm peak. Compared with fresh RBCs, lyophilized RBCs contain a significantly higher concentration of TBARS (Figure 2.5A) and probably lipid hydroperoxides that intensify the 453 nm peak; however, this assumption will need to be confirmed through further experimentation.

The reaction between t-BuOOH and TBA can create artefacts in this assay, especially when t-BuOOH is treated with TCA. This was observed as a 532 nm peak that is equivalent to a TBARS concentration of 17 nmol/L. A thorough wash after treating cells with t-BuOOH can solve this problem. However, washing can result in sample loss, particularly when studying cell components such as membrane fractions, where oxidation products (MDAs) are released into the aqueous phase. In this case, a t-BuOOH blank that is treated with TCA should be subtracted from each sample.

Most of the attempts to stabilize mammalian cells in the dried state have been performed by freeze-drying techniques with the addition of sugars, especially trehalose, as protectants [26-28]. Other protective agents include hydroxyl-ethyl starch (HES) [29, 30], bovine serum albumin (BSA) [31], human serum albumin (HSA), polyvinylpyrrolidone (PVP), and dimethyl sulfoxide (DMSO) [32]. Even with the addition of protectants, little success has been observed after lyophilization and rehydration of various mammalian cells [33]. Some plant sugars, including sucrose and trehalose, and proteins, such as BSA, may interfere with this assay [34]. A modified TBARS assay for sugar-rich plant tissue extracts was developed by Du and Bramlage [34]. Alternatively, it is recommended that the possible interference be accounted for by generating a sample blank containing the protective agents and subtracting its value from each sample.

The present data has shown that the TBARS assay can be applied to reconstituted RBC specimens. Moreover, it provided first evidence regarding the relationship between desiccation and lipid peroxidation in RBCs. The modification of the TBARS assay for use with dried mammalian cells will facilitate the study of oxidative damage that occurs

during desiccation, dry storage and rehydration of biomaterials. This assay will be used later in this study for monitoring TBARS in vacuum-dried RBCs (Chapter 4).

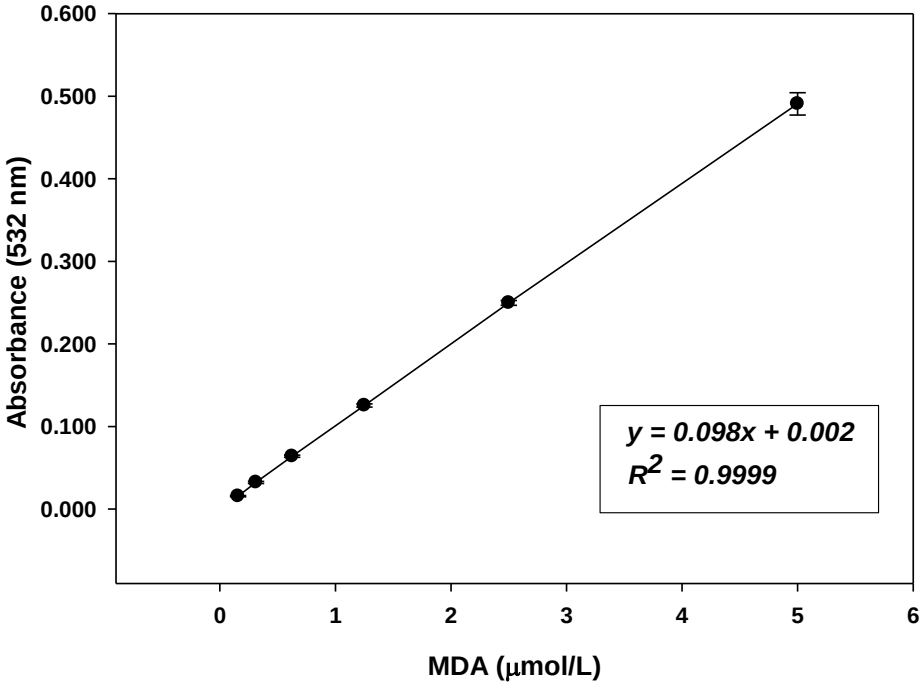

Figure 2.1 MDA standard curve. MDA standards (0.156 µmol/L to 5 µmol/L) were generated from malondialdehyde bis-(dimethyl acetal), n=6.

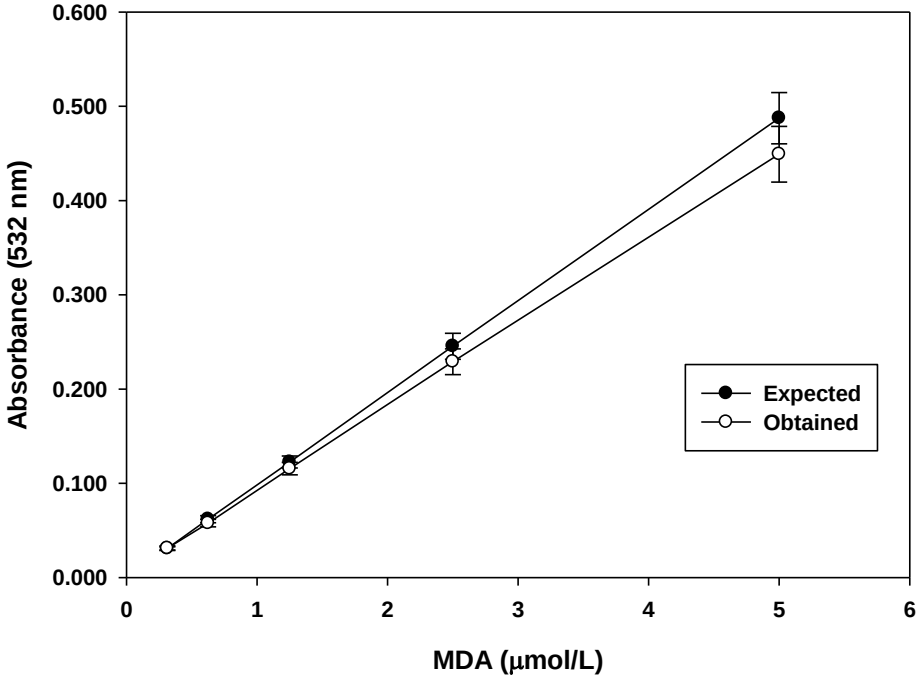

Figure 2.2. Recovery of MDA standards that have been spiked into fresh RBC suspensions. The recovery (obtained absorbance) was measured against MDA standard curves (expected absorbance). Recovery = 94 % ± 4 % (n = 7).

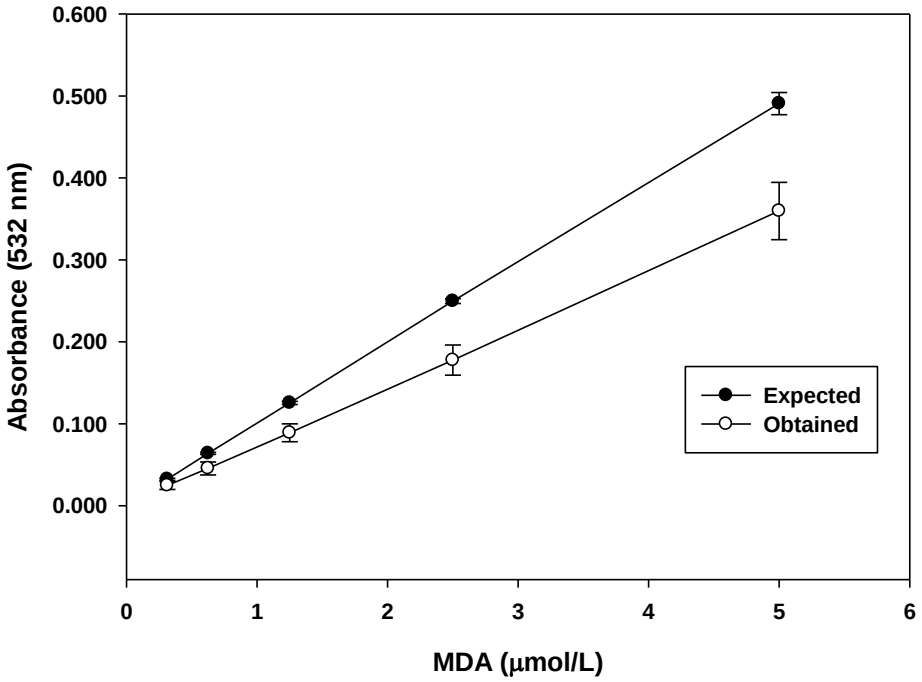

Figure 2.3. Recovery of MDA standards that have been spiked into fresh RBC suspensions and later lyophilized. The recovery (obtained absorbance) was measured against MDA standard curves (expected absorbance). Recovery = 73 % ± 9 % (n = 7).

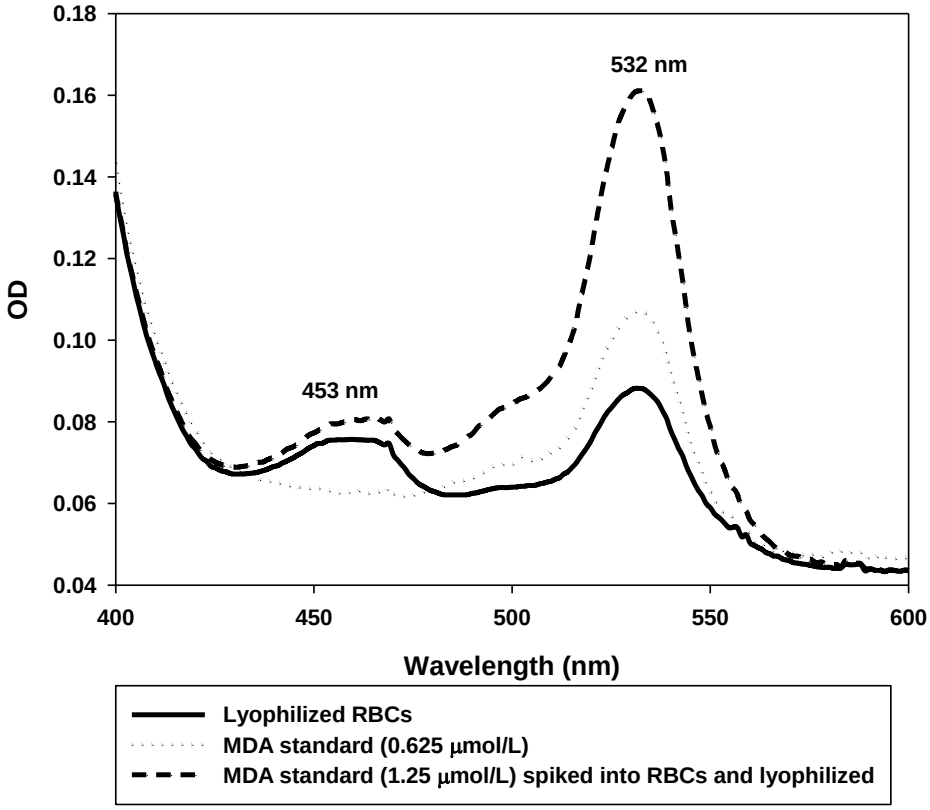

Figure 2.4. **The absorption spectra of TBARS extracted from MDA standards and lyophilized RBCs.** TBARS extracts were scanned in the range of 400 nm to 600 nm. Solid line: lyophilized RBCs; dotted line: typical absorption spectra of a MDA standard (0.625 μmol/L); dashed line: MDA standard (1.25 μmol/L) which was spiked into RBCs and later lyophilized.

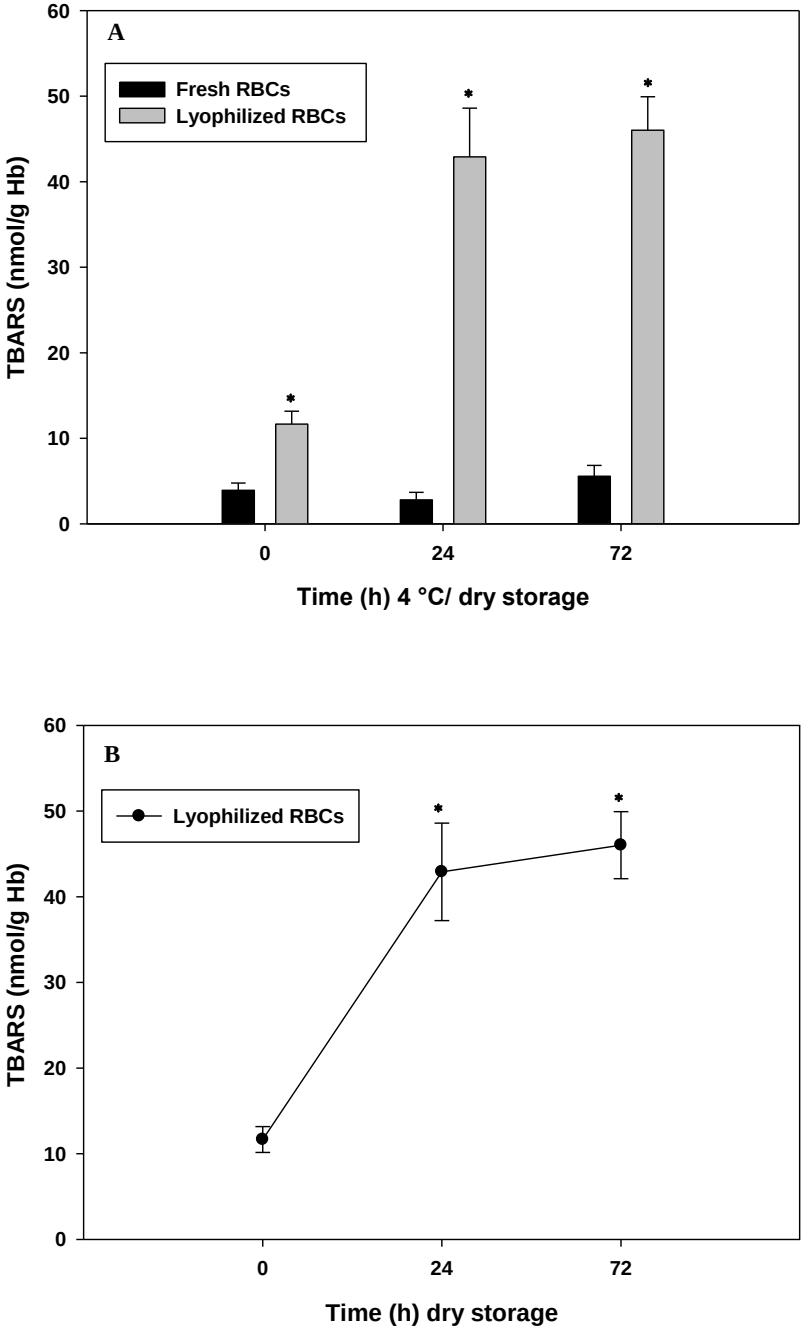

Figure 2.5 Determination of TBARS in fresh and lyophilized RBCs. A. Fresh RBC specimens in PBS were kept refrigerated (4 °C) for up to 72 h, or lyophilized overnight and rehydrated immediately (0 h), or stored at 20 °C for 24 h and 72 h prior to rehydration and assessment. Asterix symbols (*****) denote significant differences in TBARS concentration of fresh and lyophilized samples (p=0.021 at 0 h, p=0.019 at 24 h, p= 0.021at 72 h), n = 4. **B**. Formation of TBARS during dry storage (20 °C) of lyophilized RBCs (same specimens). Asterix symbol (*****) denotes a significant difference (p=0.019) between stored (24 h and 72 h) and non- stored (0 h) RBC samples (n = 4).

Table 2.1. The effects of BHT (5 mmol/L) on measured TBARS concentration (nmol/g Hb) in various RBC specimens (n=3)

Sample	Control	BHT-treated	p value
Fresh RBCs	3.3 ± 1.0	1.2 ± 0.7	0.043
Lyophilized RBCs	7.3 ± 1.4	7.4 ± 0.8	0.796
Lyophilized RBCs 72 h dry storage	38 ± 5	26 ± 4	0.050

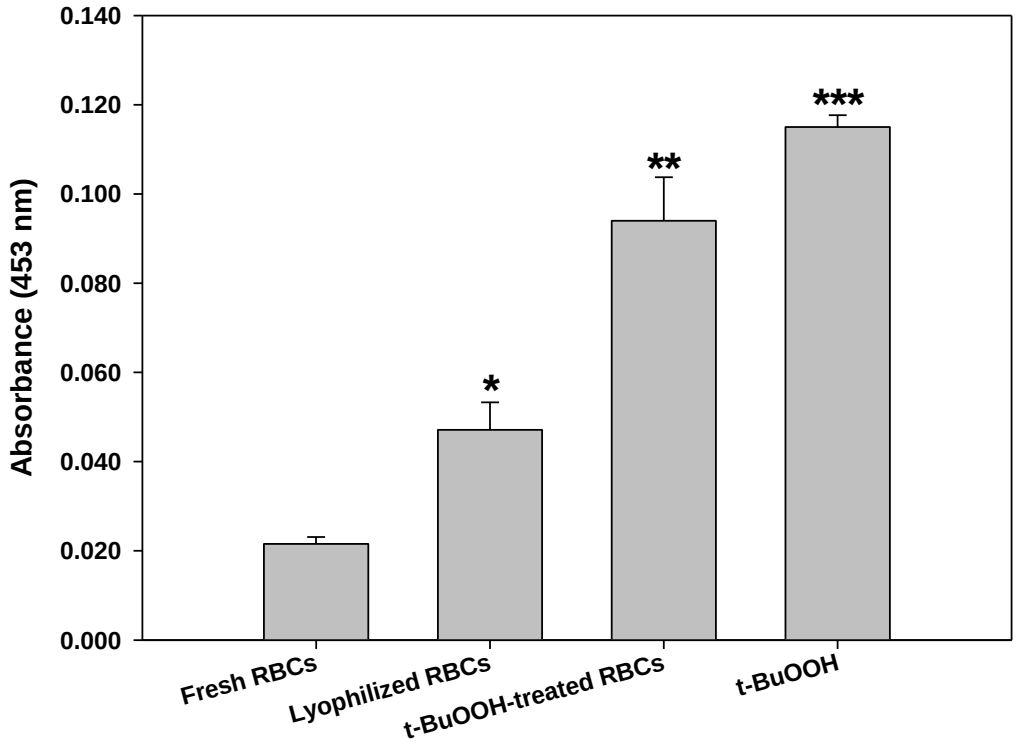

Figure 2.6 Examination of the interference caused by the 453 nm peak in the TBARS assay. The magnitude of the 453-nm peak was evaluated in TBARS from different RBC samples (HCT = 3 %) and t-BuOOH solution (2 mmol/L in PBS). Asterix symbols (***, **, *****) denote significant differences ($p<0.001$) between treatments. n=4.

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CHAPTER 3: Correlation between desiccation and oxidative injury in RBCs[4]

3.1 INTRODUCTION

The ability to survive extreme dehydration, or desiccation, has been granted to very few organisms including some species of nematodes, rotifers, lichens, yeast, bacteria, and plants [1]. Being desiccation-tolerant enables these organisms to suspend life for months, or even decades, in the absence of water [2]. Surviving the dried state is not worry-free, as various conditions can promote free radical injury and, thus, compromise the longevity of these organisms.

Abiotic stress conditions, such as high temperature, can accelerate the drying rate to a level that will compromise the ability of an organism to respond metabolically and synthesize protectants [3]. Germinating maize seeds, for example, lose their desiccation tolerance in response to rapid drying. In the absence of sufficient protection, these seeds suffered oxidative injury in terms of increased formation of ROS, as well as membrane injury due to lipid peroxidation and phospholipid de-esterification [4].

Studying the correlation between desiccation and oxidative injury at the cellular level requires the identification of cellular components capable of initiating oxidative injury. In some anhydrobiots, formation of intracellular ROS is correlated with impaired aerobic metabolism of the mitochondria; hence, reducing the respiration rate, as part of overall downregulation of metabolism, coincides with acquired resistance to desiccation-

[4] Sections of this chapter are based on a research article published as 'Kanias, T.; Acker, J. P. Mechanism of hemoglobin-induced oxidative injury in desiccated red blood cells. *Free Radic. Biol. Med.* **49**:539-547; 2010.

induced oxidative injury [5]. In contrast, human RBCs lose their mitochondria at the last stage of maturation and intracellular ROS formation is attributed primarily to Hb oxidation [6]. For that reason, Hb redox reactions have been the focus of numerous studies in the fields RBC pathologies [7, 8] and RBC *ex vivo* storage (for review see Kanias and Acker [9]).

The correlation between water loss and oxidative injury in RBCs is poorly understood. *In vivo*, RBCs are rarely exposed to dehydration except for certain pathological situations such as sickle cell disease [10]. In this case, dehydration results in increased cytoplasmatic viscosity, impaired deformability, and increased association of Hb with the membrane [11, 12]. Various factors, such as cell shrinkage and changes of oxygen pressure, can induce oxidative injury in desiccated RBCs. These factors are discussed in detail in section 1.2.4 of chapter 1.

This chapter investigates the link between desiccation and Hb oxidative injury in RBCs. It demonstrates that RBC desiccation coincides with ROS formation and Hb oxidation, as well as indicates the correlation between Hb oxidation and hemolysis.

3.2 MATERIALS AND METHODS

Reagents - All reagents are of analytical grade. Dimethyl sulfoxide (Me_2SO) was purchased from Fisher Chemical (Fair Lawn, NJ, USA). Sodium chloride, HEPES, tert-Butyl hydroperoxide solution (t-BuOOH; 70 % aqueous solution), hydrogen peroxide, sodium nitrite, 2',7'-dichlorofluorescin diacetate (DCFH-DA), and Drabkin's reagent components (Triton x-100, potassium dihydrogen phosphate, potassium cyanide and potassium ferricyanide (III)) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Preparation of human RBC suspensions - Leukocyte-reduced packed RBC units stored in saline-adenine-glucose-mannitol (SAGM) were provided by Canadian Blood Services. These units were produced from whole blood collections obtained from healthy donors. A units' age varied from 3 to 14 days (median = 7 days). The RBCs were washed twice (10 min, 3200 g, 4 °C) with HEPES buffer (20 mmol/L HEPES, 136 mmol/L sodium chloride, pH 7.4) to remove the additive solution (SAGM), the supernatant was discarded, and the pellet was suspended in HEPES buffer. The hematocrit (HCT) of each RBC suspension was determined by micro-hematocrit centrifugation (Hettich Haematokrit) and averaged 15 % ± 2 %.

Vacuum drying - RBC samples (2 mL) were placed in Falcon® 25 cm^2 non-treated cell culture flasks (Becton Dickinson and Company, NJ, USA). Flask weight (g) was determined before and after the addition of the RBC sample by using a digital balance (PG603-S, Mettler Toledo, Switzerland). Flasks containing RBC samples were placed in a vacuum oven (Fisher Isotemp 282A, Fisher Scientific Canada) and vacuum dried at 25 °C under low pressure (55 ± 5 Torr) for time lengths ranging from 24 h to 96 h. Control samples were tightly sealed (flask's cap closed and Parafilm®) and incubated (25 °C, atmospheric pressure) for the same period of time without being exposed to desiccation stress. Water loss (g) and residual moisture contents (g H_2O/ g dry weight) of vacuum dried samples were obtained by weighing the samples at the end of each drying cycle or after storage. Sample rehydration was performed in one step by adding HEPES buffer (37 °C) to vacuum-dried samples. The quantity (mL) of the added buffer corresponded to the amount of water required to obtain the original volume (2 mL). Reconstituted RBC samples were assayed for cell integrity (measured as percent hemolysis) and oxidative injury. In part of this study, vacuum dried samples have been

sealed and stored at 25 °C (atmospheric pressure) for up to 6 days prior to reconstitution. Sample water loss during storage was negligible (≤ 2-3 % of total mass). Extending the RBC exposure to dehydration stress was required to elucidate the correlation between Hb oxidation and cellular damage.

Determination of residual moisture - The residual moisture content (g H_2O/ g dry weight) of vacuum dried RBC samples was determined by gravimetric analysis [13], similar to the procedure described by Ma *et al*, [14]. Sample dry weight (g) was measured in duplicate by baking vacuum dried samples for 48 h at 80 °C. Sample water weight (g H_2O) was obtained by measuring the difference between the sample weight and the averaged dry weight of at least two identical samples. The residual moisture content is represented as g H_2O/ g dry weight. Prior to drying, RBC samples contained 15 ± 1.5 g H_2O/ g dry weight. Vacuum dried samples containing less than 1 g H_2O/ g dry weight were considered to be fully desiccated.

Determination of percent hemolysis - Percent hemolysis was determined by comparing the supernatant hemoglobin (Hb_s) to total hemoglobin (Hb_t) concentrations using Drabkin's method [15, 16]. The HCT of each sample was measured before assaying using the micro-hematocrit centrifuge. Percent hemolysis was calculated from the following equation:

$$\%\ \text{hemolysis} = \frac{(100 - \text{HCT})\text{Hb}_\text{s}}{\text{Hb}_\text{t}}$$

Determination of hemoglobin oxidation - The percentage of oxidized hemoglobin (methemoglobin) in RBC suspensions was determined by spectrophotometry using the millimolar extinction coefficients of the different hemoglobin types (oxyHb, metHb,

hemichromes) at pH 7.4 [17]. Briefly, RBC lysates were scanned from 500 nm to 700 nm while recording the absorbance values (OD) at 560 nm, 577 nm, 630 nm and 700 nm. The concentrations (mmol/L) of the different Hb types were then calculated as follows:

$$[\text{OxyHb}] = 29.8(OD_{577}-OD_{700})-9.8(OD_{630}-OD_{700})-22.2(OD_{560}-OD_{700})$$

$$[\text{MetHb}] = 7(OD_{577}-OD_{700}) +76.8(OD_{630}-OD_{700})-13.8(OD_{560}-OD_{700})$$

$$[\text{Hemichromes}] = -33.2(OD_{577}-OD_{700})-36(OD_{630}-OD_{700}) +58.2(OD_{560}-OD_{700})$$

Determination of intracellular reactive oxygen species - DCFH-DA is a membrane permeable non-fluorescence probe that is de-esterified inside the cell to yield DCFH. Oxidation of the latter by intracellular ROS results in a fluorescent product, dichlorofluorescein (DCF) [18], which can be quantified by flow cytometry. Control and reconstituted RBCs were labeled with DCFH-DA by diluting a DCFH-DA stock solution (40 mmol/L dissolved in Me_2SO) with RBC suspensions to a final concentration of 0.4 mmol/L. The RBCs were incubated for 15 min at 37 °C on a nutating mixer, washed (10 min, 1000g, 4 °C), diluted with HEPES buffer to a final concentration of $5x10^6$ cells/mL, and analyzed by flow cytometry.

Flow cytometric analyses- DCF-labeled RBCs were analysed on a flow cytometer (FACScan, BD Sciences, San Jose, CA, USA) equipped with an argon laser (488 nm). Acquisition and analysis were performed using CellQuest® software (BD Sciences). Forward scatter (FSC) was plotted against the side scatter (SSC) on a linear scale. A total of 10,000 events were collected from each sample and detected by the FL-1 channel using log amplification. The mean fluorescence intensity of the entire RBC population was obtained by CellQuest® software.

Oxidation of RBCs - RBCs oxidized with various oxidizing agents were used as positive controls throughout this study, or to validate the susceptibility of DCFH-DA to different ROS. RBC suspensions were challenged with one of three oxidizing agents (hydrogen peroxide, t-BuOOH, or sodium nitrite) at increasing concentrations of 0, 0.5, 1, 2 and 4 mmol/L. After adding the oxidizing agents, the RBC samples were placed on a nutating mixer and incubated for 60 min at 37 °C. Oxidized RBC samples were then washed, suspended with HEPES buffer, labeled with DCFH-DA, and analysed by flow cytometry. RBC oxidation by hydrogen peroxide was preceded by sodium azide treatment (1 mmol/L) to inhibit catalase activity.

Statistical analysis - A nonparametric statistical analysis was performed using commercial software (SPSS, Lead Technologies, Charlotte, NC, USA). The differences between two independent groups were analysed using Mann-Whitney U test, while Kruskal-Wallis ANOVA was used to examine the differences among multiple groups. Results are shown as the mean ± SD. Probabilities less than 0.05 were considered significant.

3.3 RESULTS

Selection of RBC vacuum-drying conditions - This section summarizes experimental data (Table 3.1), which helped defining the drying conditions that would be used later in this study. The optimization protocol is based on three parameters: drying time (h), temperature (°C) and pressure (Torr). Each of these parameters can significantly affect the drying rate and consequently, cell integrity. Exposing RBC specimens to extremely low pressure (1 Torr) and 37 °C was lethal (100 % hemolysis after 1 h) due to fast drying rate and low residual moisture content (0.8 g H_2O/g dry weight). Drying at extremely low pressures (≤1 Torr) drastically decreases the boiling point of liquids and,

therefore, is not suitable for RBC suspensions. Under these conditions, boiling of RBC specimens started at about 20 Torr. For that reason, the chamber pressure in subsequent experiments was set to higher values of 50 to 70 Torr.

Setting the vacuum-oven temperature to 37 °C was ineffective in terms of fast drying rate and low cell integrity (Table 3.1). Under these conditions, sample water content dropped from about 15 g H_2O/g dry weight (fresh) to 4.5 g H_2O/g dry weight in 1 h. After 20 h, samples were fully desiccated and hemolysed. Setting the chamber to 25 °C and 55 Torr has extended the drying time to about 96 h. Slowing the drying rate provided a wider dynamic range, which improved our ability to detect changes in the RBC oxidative state during desiccation. These conditions have been chosen for the entire study.

Desiccation-induced Hb oxidation and hemolysis in vacuum-dried RBCs - The first set of experiments (Figures 3.1 to 3.3) represents the extent of Hb oxidation during RBC desiccation and its correlation to hemolysis. Exposing RBCs to our desiccation system (vacuum-drying at 55 ± 5 Torr, 25 °C) resulted in metHb formation (Figure 3.1) and hemolysis (Figure 3.2). Both stress indicators initially appeared at residual moisture contents of approximately 5 g H_2O/g dry weight. At this moisture content, metHb levels increased from ≤ 1 % to 1.9 %. The rate of metHb formation increased as a function of the residual moisture content reaching about 15 % at 0.7 g H_2O/g dry weight (Figure 3.1).

Water loss has been also correlated with a significant increase in hemolysis levels (Figure 3.2). Similar to metHb formation, the incidence of cell death intensified at lower residual moisture contents, reaching a maximum (100 % hemolysis) at 0.7 g H_2O/g dry weight. The high levels of hemolysis (80 % to 100 %) observed at the range of about 1 g

H_2O/g dry weight are likely the consequence of physical and mechanical damage due to fusion, surface adhesion, and osmotic shock. These factors significantly affected our ability to study the role of oxidative injury in mediating cellular damage. Therefore, further experiments did not exceed the range of 2 g H_2O/g dry weight, which yielded higher levels of cell integrity.

The correlation between Hb oxidation and hemolysis during desiccation is depicted in Figure 3.3. The sigmoid-shaped curve obtained from a five parameter polynomial fit (R=0.982) indicates a remarkable increase of hemolysis levels (10 % to 80 %) at the range of 4 % to 10 % metHb. This range corresponds to residual moisture contents of about 4 g H_2O/g dry weight to 1.2 g H_2O/g dry weight.

DCF sensitivity to oxidizing agents - The ability to monitor ROS formation in RBCs was tested by treating DCF-labeled RBCs with different oxidizing agents. Flow cytometric analysis revealed that hydrogen peroxide was the most potent oxidizing agent compared with t-BuOOH and sodium nitrite (Figure 3.4). Hydrogen peroxide caused a significant increase in DCF fluorescence ($p=0.003$) at the lowest tested dosage of 0.5 mmol/L; conversely, no changes in DCF fluorescence were observed with t-BuOOH or sodium nitrite. Significant differences ($p \leq 0.001$) between the three oxidizing agents were noticeable at the higher tested dosages of 1, 2 and 4 mmol/L. Hydrogen peroxide and t-BuOOH caused an increase in DCF-fluorescence in a dose response manner, while sodium nitrite did not induce changes in DCF fluorescence at any of the tested dosages. Moreover, no significant differences ($p=0.615$) were observed between non-oxidized DCF-labeled RBCs (0 mmol/L) and sodium nitrite treated (0.5 to 4 mmol/L) RBCs.

ROS formation during RBC desiccation - Flow cytometric analysis of DCF-labeled RBCs confirms the formation of intracellular ROS in control and reconstituted RBCs (Figure 3.5). Hydrogen peroxide treatment (2 mmol/L) of DCF-labeled RBCs is used as a reference (positive control; Figure 3.5). ROS formation in reconstituted RBCs is affected by the drying time (Figure 3.5), and the samples' residual moisture content (Figure 3.6). The ratio between vacuum-drying and storage (Figure 3.5) denotes the number of days, in which a sample was exposed to vacuum-drying within a period of 7 day storage. For instance, 4/7 means 4 days of vacuum drying followed by 3 day storage at 25 °C.

Seven day storage of RBCs under atmospheric pressure (controls) doubled the levels of baseline ROS (Figure 3.5; 0/7 versus fresh, respectively; $p<0.001$). ROS levels in RBCs subjected to 1 day drying and 6 day storage at relatively high residual moisture content (10 g H_2O/g dry weight) were not significantly ($p=0.885$) different than that of the controls (Figure 3.5; 1/7 versus 0/7, respectively). On the other hand, a significant increase ($p<0.01$) in ROS levels was observed in RBCs subjected to longer drying periods (Figure 3.5; 3/7 and 4/7) and storage under lower residual moisture contents (6 to 2 g H_2O/g dry weight; Figure 3.6).

3.4 DISCUSSION

Drying method - Vacuum-drying has enabled us to study the extent of desiccation-induced oxidative injury in RBCs under specific conditions (25 °C, 55±5 Torr). Selection of the drying conditions was driven by the desire to study the relationship between RBC desiccation and oxidative injury rather than creating a protocol for improved preservation of RBCs in the dried state. Thus, additives such as cellular protectants were not added to the drying buffer. HEPES was selected as the buffer of

choice for its superior acid-base regulation during cryopreservation of cell cultures and organs [19]. Sustaining physiological pH during desiccation stress was important for this study, as intracellular acidosis can adversely affect cellular recovery through various mechanisms including modification of protein activity, and metabolic blockage of glycolysis [19].

Choosing different drying conditions (temperature, pressure) for studying the relationship between RBC desiccation and oxidation can alter the extent of the oxidative injury, but is unlikely to eliminate it. Moreover, RBC oxidative injury during desiccation is not unique to our drying method, but rather a wider phenomenon, which has been documented with other methods including freeze-drying, where reconstituted RBCs contained about 50 % metHb [20] (for review see section 1.2.4 of Chapter 1).

Hemoglobin oxidation reaches a maximum at critical residual moisture content - In our desiccation system, the RBC hemoglobin can resist autoxidation up to a range of about 5 g H_2O/g dry weight (Figure 3.1), which is concurrent with initiation of hemolysis (Figure 3.2). The correlation between these two phenomena, represented by a sigmoid curve (Figure 3.3), reveals a critical residual moisture zone (4 to 1.2 g H_2O/g dry weight), where the increase in Hb oxidation and hemolysis levels are maximal (2.5-fold and 8-fold, respectively). This zone implies that drastic changes in the Hb oxidative state occur in RBC samples that are partially dehydrated, rather than samples that are relatively dry (1.0 to 0.7 g H_2O/g dry weight). This finding has an important implication regarding the role hemoglobin plays in mediating cellular injury during RBC desiccation. Subsequent findings, which will be discussed in Chapter 4, will demonstrate how counteracting hemoglobin redox reactions in RBC samples that are partially dehydrated can improve cell integrity.

DCF sensitivity to oxidizing agents - Treating DCF-labeled RBCs with different oxidizing agents revealed that hydrogen peroxide and t-BuOOH caused a dose-response increase in DCF fluorescence while sodium nitrite did not (Figure 3.4). Characterizing the sensitivity of the DCF probe to different oxidizing agents provides a clue regarding the sort of ROS that can be generated during desiccation and dry storage of RBCs. Hydrogen peroxide, for example, can be produced in RBCs by dismutation of superoxide anions (Equation 2, Chapter 1). t-BuOOH is an analog of lipid hydroperoxides, which can be formed intracellularly by decomposition of oxidized membrane lipids [21]. Sodium nitrite treatment of RBCs can generate ROS and nitric oxide species (NOS) through its reactions with hemoglobins [22, 23]. Chapter 4 will discuss the use of sodium nitrite in regard to production of metHb RBCs.

ROS formation during red blood cell desiccation is a pre-hemolytic event - One concept, frequently debated in the studies of desiccation tolerance, is whether oxidative injury is a cause or a consequence of organism death. The argument is that free radical injury can take place before or after the time of death [24]. In the case of vacuum-dried RBCs, it was not clear whether hemoglobin oxidation promotes hemolysis or vice versa, as hemoglobin is readily oxidized once released from hemolysed RBCs. Flow cytometric analysis of DCF fluorescence enabled us to detect intracellular ROS in vacuum-dried RBCs.

Our study of DCF-labeled RBCs demonstrated that DCF fluorescence is generated primarily by intact cells, whereas signal loss is observed in vacuum-dried samples, which contained high levels of hemolysis (70 % to 100 %). Attenuation of DCF fluorescence in these samples can be explained by leakage of the probe in response to hemolysis, or by the loss of intracellular esterase activity, which is required for DCFH-

DA hydrolysis into DCFH [18]. The observation that DCF fluorescence is produced by intact cells suggests that at least part of the oxidative injury monitored in reconstituted RBCs (Figures 3.5 and 3.6) took place prior to hemolysis (Figures 3.5 and 3.6). Chapter 4 will examine whether the observed ROS are a consequence of Hb oxidation by vacuum-drying RBCs with reduced capacity to Hb oxidation.

The aforementioned data supports the general hypothesis by several means. It provides evidence regarding the correlation between desiccation and oxidative injury in RBCs, as well as demonstrates the correlation between Hb oxidation and hemolysis in desiccated RBCs. It defines the range of residual moisture content where Hb oxidation is maximal, illustrates the correlation between water loss and ROS formation, and suggests that the oxidative injury preceded hemolysis. These findings are in agreement with previous observations regarding Hb oxidation during desiccation. Török *et al*, [20, 25] demonstrated that lyophilization and dry storage of trehalose-loaded RBCs is accompanied with a dramatic increase of intracellular metHb. The next chapter will take a further step in understanding the correlation between Hb oxidation and hemolysis by inhibiting Hb redox reactions during desiccation.

Table 3.1. **Selection of vacuum-drying conditions for RBC oxidative injury study.** RBC samples (15 ± 2 % HCT) in HEPES buffer were vacuum-dried under defined conditions (see below) and rehydrated with HEPES buffer as described in section 3.2. n= 3-6.

Drying time (h)	**Oven temperature** (°C)	**Pressure** (Torr)	**Hemolysis** (%)	**MetHb** (%)	**Residual moisture** (g H_20/g dry weight)
1	37±1	<1	100	30	0.8
1	37±1	65±5	1.0±0.1	<1	4.5
20	37±1	65±5	100	75	0.2
24	25±1	55±5	0.5±0.1	<1	8.6±0.5
48	25±1	55±5	0.8±0.2	<1	7.6±1.1
72	25±1	55±5	2.2±0.6	1.7±0.4	4.5±0.6
96	25±1	55±5	92±4	13±1	0.8±0.1

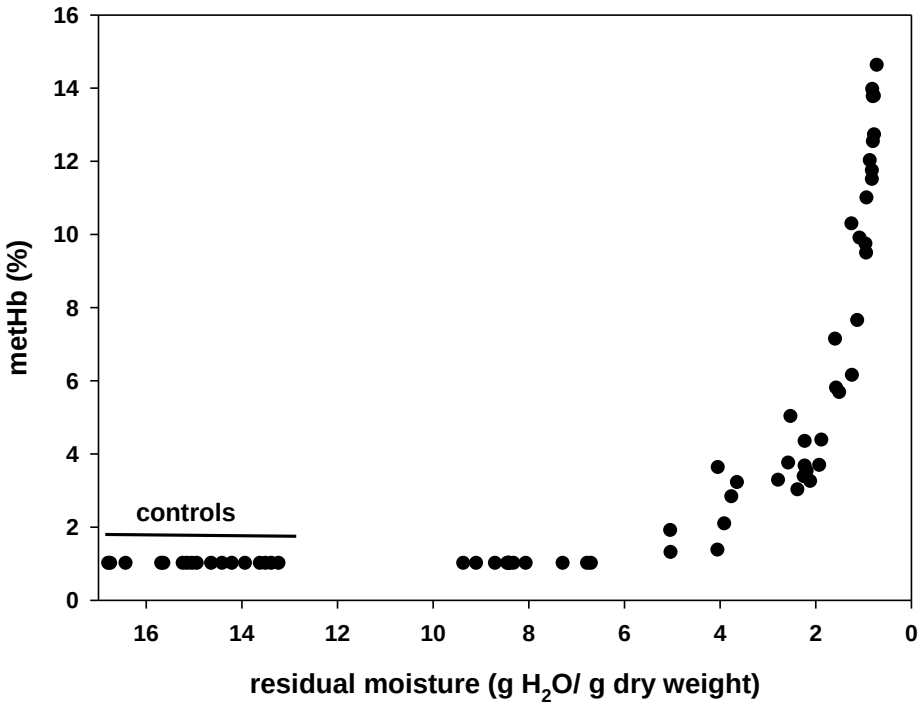

Figure 3.1. Rate of Hb oxidation (% metHb) as a function of the residual moisture content in vacuum-dried RBCs. RBC samples were vacuum-dried (55 ± 5 Torr, 25 °C, 1-4 days) to various residual moisture contents. Control RBCs were incubated (25 °C, atmospheric pressure) for the same period of time without being exposed to desiccation. The curves represent a total of 66 individual samples (control and vacuum-dried) obtained from 7 different red blood cell units.

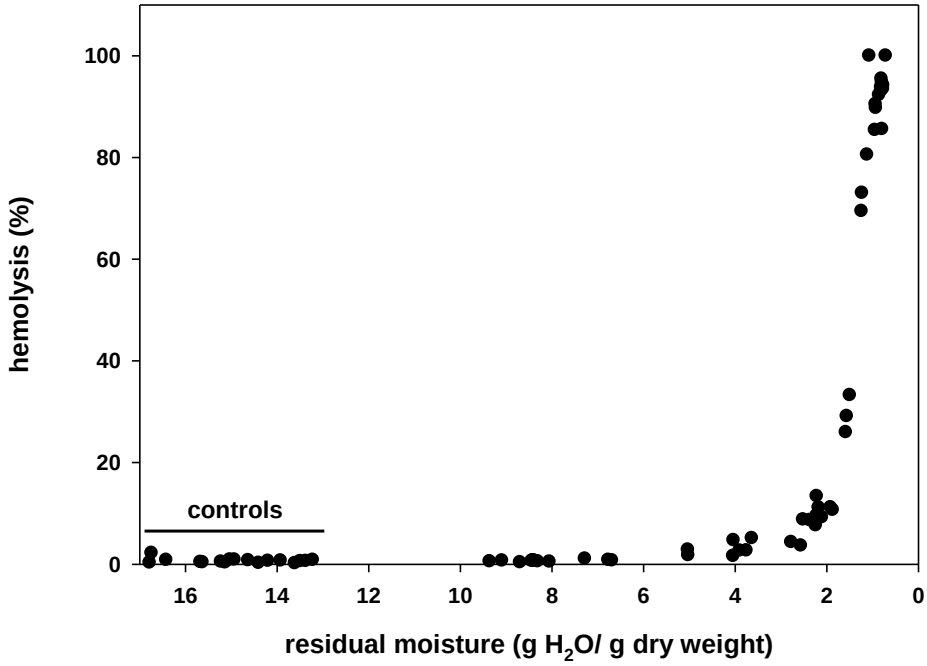

Figure 3.2. RBC hemolysis (%) as a function of the residual moisture content in vacuum-dried RBCs. RBC samples were vacuum-dried (55 ± 5 Torr, 25 °C, 1-4 days) to various residual moisture contents. Control RBCs were incubated (25 °C, atmospheric pressure) for the same period of time without being exposed to desiccation. The curves represent a total of 66 individual samples (control and vacuum-dried) obtained from 7 different red blood cell units.

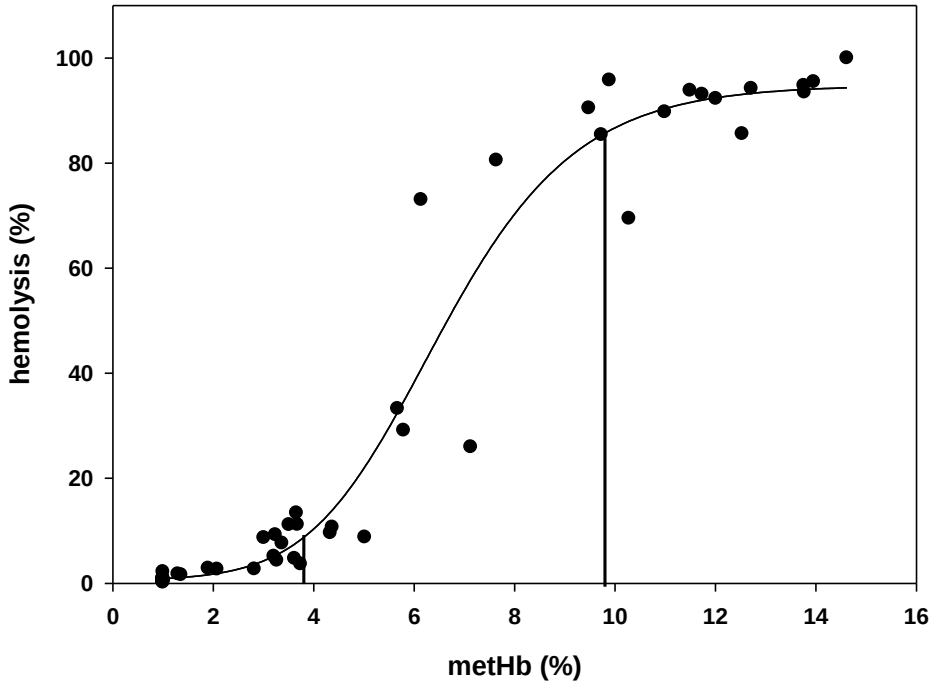

Figure 3.3. Correlation between Hb oxidation and hemolysis in vacuum-dried RBCs. The correlation was obtained by plotting the rate of Hb oxidation (Figure 3.1) versus the rate of hemolysis (Figure 3.2) using a 5 parameter polynomial fit (R=0.982). The linear zone of the sigmoid curve (defined by two vertical lines) corresponds to residual moisture contents in the range of about 4 to 1.2 g H_2O/g dry weight.

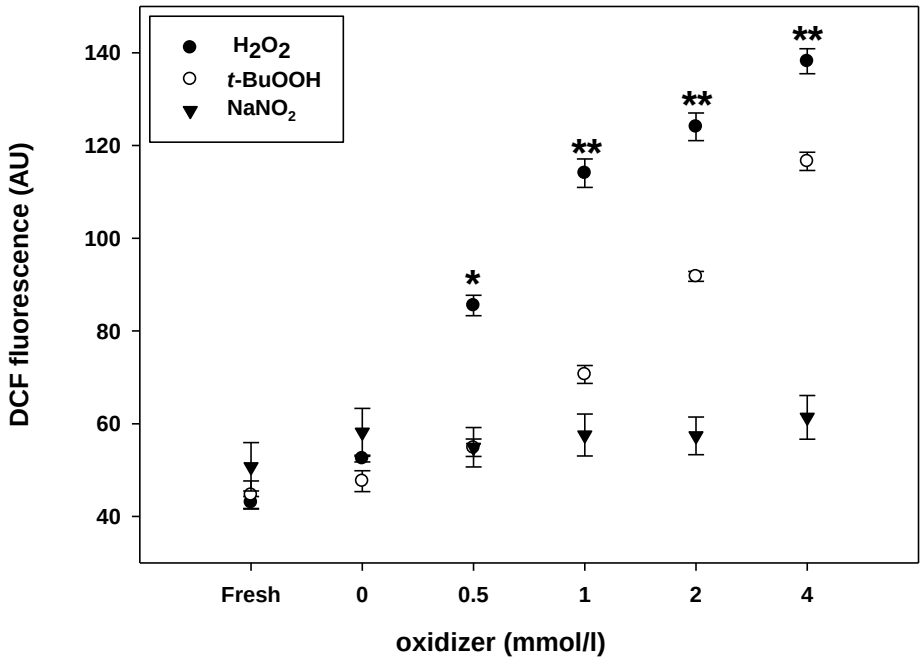

Figure 3.4. RBC ROS formation in response to different oxidizing agents. Flow cytometric analysis of DCF-labeled RBCs, which were treated with different oxidizing agents (hydrogen peroxide (H_2O_2), t-BuOOH, sodium nitrite ($NaNO_2$); see section 3.2 for details) at 0, 0.5,1, 2 and 4 mmol/L. Error bars represent standard errors. One asterisk symbol (*) denotes significant differences (p=0.003) between H_2O_2-treated RBCs and t-BuOOH/ $NaNO_2$-treated RBCs at 0.5 mmol/L. Two asterisk symbols (**) denote significant differences (p=0.001) among the three oxidizing agents at 1, 2 and 4 mmol/L. n=6 collected from three independent experiments with three different RBC units.

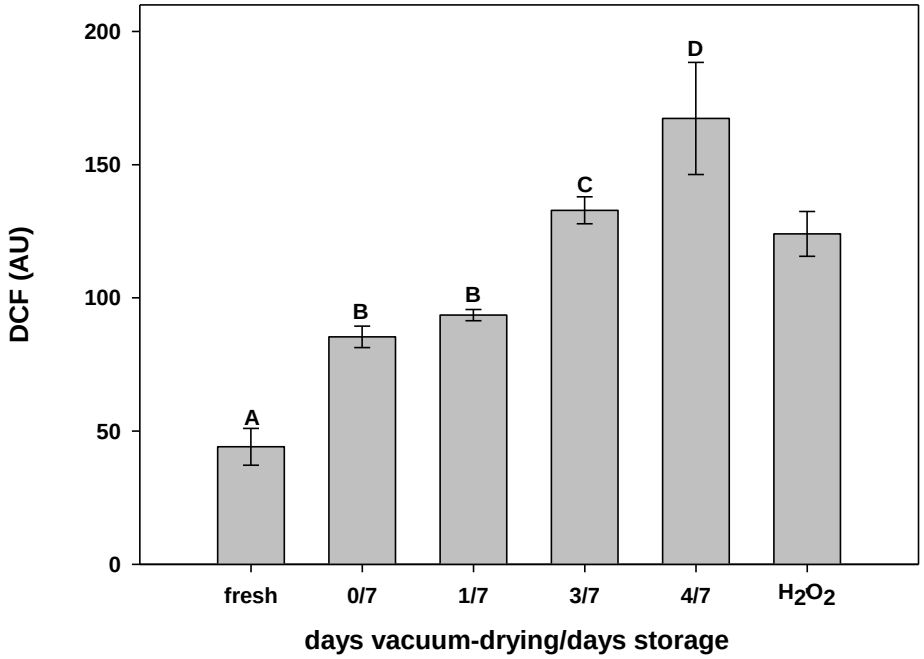

Figure 3.5. RBC ROS formation as a function of the drying period. ROS formation derives from flow cytometric analysis of DCF fluorescence (arbitrary units) monitored in fresh, stored or vacuum-dried RBCs. Data is represented as exposure time (days) to vacuum-drying within a period of 7 day storage at 25 °C. Control RBC samples were incubated for 7 days without being exposed to vacuum-drying (0/7). Positive control has been obtained by treating RBC suspensions with H_2O_2 (2 mmol/l). Error bars represent standard deviation. A,B,C and D denote significant differences ($p < 0.05$) in mean fluorescence intensity levels between four groups: A, fresh; B, control (0/7) and 1 day vacuum-drying (1/7); C, 3 day vacuum-drying (3/7); D, 4 day vacuum-drying (4/7). n= 4-12.

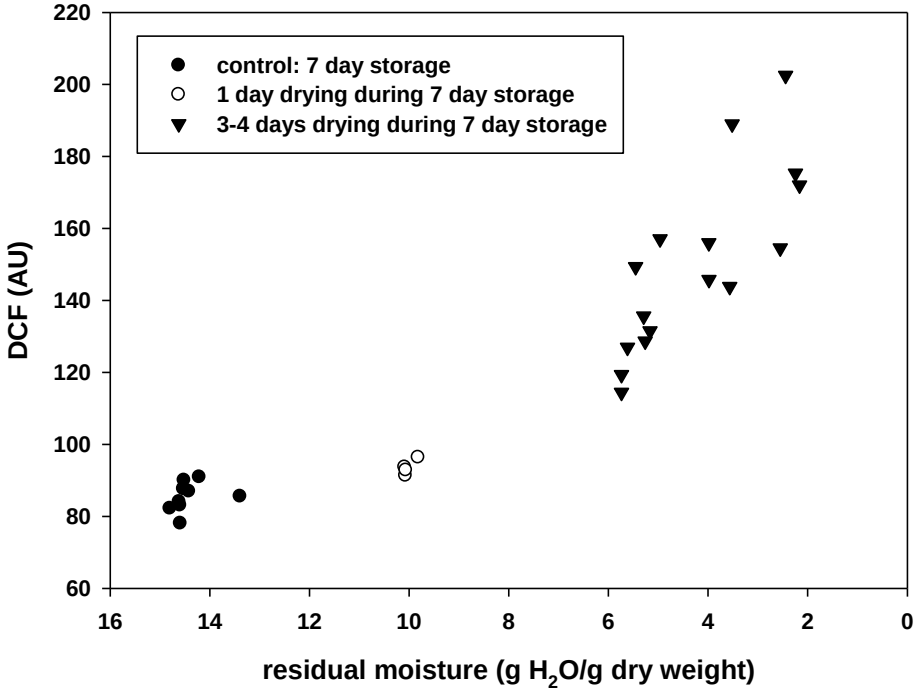

Figure 3.6. RBC ROS formation as a function of the residual moisture content. ROS formation derives from flow cytometric analysis of DCF fluorescence (arbitrary units) monitored in control (7 day storage at 25 °C) and vacuum-dried RBCs (total of 27 samples).

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CHAPTER 4: Mechanism of hemoglobin-induced oxidative injury in desiccated RBCs[5]

4.1 INTRODUCTION

The previous chapter provided evidence regarding the correlation between Hb oxidation and hemolysis in desiccated RBCs. This chapter elucidates the circumstances by which Hb oxidation promotes hemolysis in vacuum-dried RBCs. As reviewed in section 1.2.2 of Chapter 1, the autoxidation of oxyHb ($HbFe(II)O_2$) yields two potentially toxic products (Equation 4.1) that can significantly affect the recovery of vacuum-dried RBCs. The first product, superoxide anion ($O_2^{\cdot-}$) [1], is capable of participating in redox reactions, which can lead to the formation of highly toxic ROS including hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) [2]. ROS promote hemolysis through unrestricted oxidation of various cellular components including Hb, cytoskeleton proteins and membrane lipids.

Autoxidation of hemoglobin

$$\mathbf{HbFe(II)O_2 \xrightarrow{autoxidation} HbFe(III)+O_2^{\cdot-}} \qquad \mathbf{(4.1)}$$

MetHb (HbFe(III)), the second product of Hb oxidation, is incapable of reversibly binding oxygen at physiological partial oxygen pressure [3], hence reducing the capacity of the red blood cell to deliver oxygen. Oxidative denaturation of metHb can be induced by its conversion into unstable structures of hemoglobins, such as hemichromes. Denatured Hb products, particularly free heme, can promote membrane

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damage and hemolysis through mechanisms including lipid peroxidation, and oxidation of protein thiol groups [4].

This chapter's goal is to verify whether desiccation-induced Hb oxidation is directly involved with compromised cell integrity. It focuses on both Hb oxidation products (ROS, metHb) and demonstrates that inhibition of Hb redox reactions during desiccation can significantly affect cell integrity post reconstitution.

4.2 MATERIALS AND METHODS

Reagents - All reagents are of analytical grade. Dimethyl sulfoxide (Me_2SO) was purchased from Fisher Chemical (Fair Lawn, NJ, USA). Sodium chloride, HEPES, tert-Butyl hydroperoxide solution (t-BuOOH; 70 % aqueous solution), hydrogen peroxide, potassium phosphate monobasic, potassium phosphate dibasic, sodium dithionite, 2',7'-dichlorofluorescin diacetate (DCFH-DA), and Drabkin's reagent components (Triton x-100, potassium dihydrogen phosphate, potassium cyanide and potassium ferricyanide (III)) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Preparation of human RBC suspensions - Leukocyte-reduced packed RBC units stored in saline-adenine-glucose-mannitol (SAGM) were provided by Canadian Blood Services. These units were produced from whole blood collections obtained from healthy donors. A units' age varied from 3 to 14 days (median = 7 days). The RBCs were washed twice (10 min, 3200 g, 4 °C) with HEPES buffer (20 mmol/L HEPES, 136 mmol/L sodium chloride, pH 7.4) to remove the additive solution (SAGM), the supernatant was discarded, and the pellet was suspended in HEPES buffer. The hematocrit (HCT) of each RBC suspension was determined by micro-hematocrit centrifugation (Hettich Haematokrit) and averaged 15 % ± 2 %.

Correlation between Hb oxidation and cellular damage during desiccation - The involvement of Hb oxidation in mediating cellular damage during desiccation was tested by two approaches: 1. minimizing the probability of Hb oxidation during desiccation by blocking the Hb oxygen binding site. This procedure was achieved by treating RBC suspensions with carbon monoxide (CO) prior to vacuum drying and storage. 2. Inducing a near complete oxidation of the RBC Hb (95 ± 3 % metHb) prior to vacuum drying and storage. This procedure was achieved by treating RBC suspensions with sodium nitrite.

Production of carboxyHb RBCs: RBC suspensions, prepared as described under 'preparation of human RBC suspensions', were placed on ice and CO was bubbled through the samples for 60 min. Following CO treatment, the RBC suspensions were washed twice (10 min, 3200 g, 4 °C) and the packed RBC were suspended with HEPES buffer. This procedure yields RBCs containing approximately 90 % carboxyHb. Percentage carboxyHb was determined by spectrophotometry as described by Beutler and West [5].

Production of metHb RBCs: RBC suspensions, prepared as described under 'preparation of human RBC suspensions', were treated with sodium nitrite (8 mmol/L) for 30 min at 20 °C with mild agitation. The RBC suspensions were then washed twice (10 min, 3200 g, 4 °C) and the packed RBCs were suspended with HEPES buffer. This procedure yields about 95 % metHb. Hemichrome formation was not detected in fresh metHb RBC suspensions. Cell integrity of washed metHb RBCs was compared with that of fresh untreated RBC suspension (same donor). Flow cytometric analysis of DCF fluorescence (arbitrary units) revealed no significant differences between the two RBC groups (40 ± 1 for untreated RBCs versus 40 ± 2 for metHb RBCs). Hemolysis levels were also similar in both RBC groups and measured less than 1 %.

Vacuum drying - RBC samples (2 mL) were placed in Falcon® 25 cm^2 non-treated cell culture flasks (Becton Dickinson and Company, NJ, USA). Flask weight (g) was determined before and after the addition of the RBC sample by using a digital balance (PG603-S, Mettler Toledo, Switzerland). Flasks containing RBC samples were placed in a vacuum oven (Fisher Isotemp 282A, Fisher Scientific Canada) and vacuum dried at 25 °C under low pressure (55 ± 5 Torr) for time lengths ranging from 24 h to 96 h. Control samples were tightly sealed (flask's cap closed and Parafilm®) and incubated for the same period of time without being exposed to desiccation stress (25 °C, atmospheric pressure). At the end of each drying cycle, samples were weighed, sealed (flask's cap closed and Parafilm®) and stored at 25 °C (atmospheric pressure) for up to 6 days. At the end of the incubation period, control and vacuum-dried RBC samples were weighed for determining the residual moisture content (g H_2O/ g dry weight). Sample water loss during storage was negligible (≤ 2-3 % of total mass). Vacuum-dried samples were rehydrated in one step by adding HEPES buffer (37 °C). The quantity (mL) of the added buffer corresponded to the amount of water required to obtain the original volume (2 mL).

Determination of residual moisture - The residual moisture content (g H_2O/ g dry weight) of vacuum dried RBC samples was determined by gravimetric analysis [6], similar to the procedure described by Ma *et al*, [7]. Sample dry weight (g) was measured in duplicates by baking vacuum dried samples for 48 h at 80 °C. Sample water weight (g H_2O) was obtained by measuring the difference between the sample weight and the averaged dry weight of at least two identical samples. The residual moisture content is represented as g H_2O/ g dry weight. Prior to drying, RBC samples contained 15 ± 1.1 g

H_2O/ g dry weight. Vacuum dried samples containing less than 1 g H_2O/ g dry weight were considered to be fully desiccated.

Determination of percent hemolysis - Percent hemolysis was determined by comparing the supernatant hemoglobin (Hb_s) to total hemoglobin (Hb_t) concentrations, using Drabkin's method [8, 9]. The HCT of each sample was measured before assaying using the micro-hematocrit centrifuge. Samples containing carboxyHb required longer reaction time with the assay's reagents; therefore, they were incubated for 2 h prior to analysis. Percent hemolysis was calculated from the following equation:

$$\%\ \text{hemolysis} = \frac{(100 - \text{HCT})\text{Hb}_\text{s}}{\text{Hb}_\text{t}}$$

Determination of hemoglobin oxidation - The percentage of oxidized hemoglobin (methemoglobin) in RBC suspensions was determined by spectrophotometry using the millimolar extinction coefficients of the different Hb types (oxyhemoglobin, methemoglobin, hemichromes) at pH 7.4 [10]. Briefly, RBC lysates were scanned from 500 nm to 700 nm while recording the absorbance values (OD) at 560 nm, 577 nm, 630 nm and 700 nm. The concentrations (mmol/L) of the different Hb types were then calculated as follows:

$[\text{OxyHb}] = 29.8(OD_{577}\text{-}OD_{700})\text{-}9.8(OD_{630}\text{-}OD_{700})\text{-}22.2(OD_{560}\text{-}OD_{700})$

$[\text{MetHb}] = 7(OD_{577}\text{-}OD_{700}) + 76.8(OD_{630}\text{-}OD_{700})\text{-}13.8(OD_{560}\text{-}OD_{700})$

$[\text{Hemichromes}] = -33.2(OD_{577}\text{-}OD_{700})\text{-}36(OD_{630}\text{-}OD_{700}) + 58.2(OD_{560}\text{-}OD_{700})$

This method is not suitable for measuring metHb in RBC samples containing high levels of carboxyHb. In that part of the study, percent metHb was measured by a different

spectrophotometric method, as described by Sato [11]. This method is based on the phenomenon that metHb maximum absorbance at 630 nm disappears in response to addition of cyanide. MetHb concentration is determined as the ratio of the absorbance changes induced by cyanide before and after the addition of potassium ferricyanide [11].

Determination of lipid peroxidation - Membrane lipid peroxidation was measured by TBARS as described in Chapter 2.

Determination of intracellular reactive oxygen species - Formation of intracellular ROS was monitored by flow cytometry using the DCFH-DA probe. Control and reconstituted RBCs with different types of Hbs (oxyHb, metHb and carboxyHb) were labeled with DCFH-DA by diluting a DCFH-DA stock solution (40 mmol/L dissolved in Me_2SO) with each RBC sample to a final concentration of 0.4 mmol/L. The RBCs were incubated for 15 min at 37 °C on a nutating mixer, washed (10 min, 1000g, 4 °C), diluted with HEPES buffer to a final concentration of $5x10^6$ cells/mL, and analyzed by flow cytometry.

Flow cytometric analyses - DCF-labeled RBCs were analysed on a flow cytometer (FACScan, BD Sciences, San Jose, CA, USA) equipped with an argon laser (488 nm). Acquisition and analysis were performed using CellQuest® software (BD Sciences). Forward scatter (FSC) was plotted against the side scatter (SSC) on a linear scale. A total of 10,000 events were collected from each sample and detected by the FL-1 channel using log amplification. The mean fluorescence intensity of the entire RBC population was obtained by CellQuest® software.

Oxidation of oxyHb and carboxyHb RBCs - CarboxyHb RBC susceptibility to oxidation was tested by oxidative challenge using hydrogen peroxide [2 mmol/L] or t-

BuOOH [2 mmol/L]. The increase in ROS formation was compared to that of oxyHb RBCs, which were oxidized in the same manner. After adding the oxidizing agents, the RBC samples were placed on a nutating mixer and incubated for 60 min at 37 °C. Oxidized RBC samples were then washed, suspended with HEPES buffer, labeled with DCFH-DA, and analysed by flow cytometry. RBC oxidation by hydrogen peroxide was preceded by sodium azide treatment (1 mmol/L) to inhibit catalase activity.

Statistical analysis - A nonparametric statistical analysis was performed using commercial software (SPSS, Lead Technologies, Charlotte, NC, USA). The differences between two independent groups were analysed using Mann-Whitney U test, while Kruskal-Wallis ANOVA was used to examine the differences among multiple groups. Results are shown as the mean ± SD. Probabilities less than 0.05 were considered significant.

4.3 RESULTS

Vacuum-drying of carboxyHb RBCs - The involvement of Hb oxidation in promoting hemolysis during RBC desiccation was tested by modifying the Hb affinity to oxygen. Blocking the Hb oxygen binding site by CO treatment (carboxyHb RBCs) significantly ($p<0.0001$ at all defined moisture contents) reduced metHb formation in control and vacuum-dried carboxyHb RBCs compared with oxyHb RBCs (Figure 4.1). Despite the lower levels of metHb, carboxyHb RBCs suffered higher levels of hemolysis (Figure 4.2; $p<0.05$ at all defined moisture contents) and intracellular ROS (Figure 4.3; $p<0.05$ at 5.4 ± 0.2 g H_2O/g dry weight and 2.4 ± 0.4 g H_2O/g dry weight) compared with control and vacuum-dried oxyHb RBCs.

The differences between the RBC groups were further characterized using an oxidation test. CarboxyHb RBCs and oxyHb RBCs were challenged with two different oxidizing agents: H_2O_2 that is naturally formed in red blood cells, and t-BuOOH, a lipid hydroperoxide analog. ROS formation in response to H_2O_2 treatment [2 mmol/L] was about 30 % lower ($p=0.009$) in carboxyHb RBCs than that of oxyHb RBCs (Figure 4.4). Conversely, carboxyHb RBCs exhibited similar susceptibility to that of oxyHb RBCs when challenged with t-BuOOH [2 mmol/L].

Vacuum-drying of metHb RBCs – Transforming the RBC Hb to metHb (metHb RBCs) prior to vacuum-drying allowed us to study the potential role that metHb plays in mediating cellular injury during RBC desiccation. Spectrophotometric analysis of hemoglobins from reconstituted oxyHb RBCs and metHb RBCs confirmed that the latter contained between 80 % to 90 % higher levels of metHb (Figure 4.5). We did not detect hemichromes in either RBC group. ROS levels in vacuum-dried metHb RBCs were either similar or higher than that of oxyHb RBCs (Figure 4.6). Control metHb RBCs (15 ± 0.7 g H_2O/g dry weight) contained significantly ($p<0.001$) higher levels of ROS compared with oxyHb RBCs (Figure 4.6).

Converting the RBC Hb to metHb significantly improved cell integrity following vacuum-drying and storage of samples, which contained medium levels of residual moisture contents (5.2 ± 0.2 or 3.7 ± 0.2 g H_2O/g dry weight) (Figure 4.7). These metHb RBC samples contained significantly ($p=0.01$ and 0.02, respectively) lower levels of hemolysis than that of equivalent oxyHb RBC samples (Figure 4.7). A decrease of 37 % in hemolysis levels occurred at both residual moisture contents (5.2 ± 0.2 and 3.7 ± 0.2 g H_2O/g dry weight). Conversely, no significant differences between the two RBC groups

have been observed at higher (15 ± 0.7 and 9.9 ± 0.2 g H_2O/g dry weight) or lower (2.4 ± 0.4 g H_2O/g dry weight) residual moisture contents (Figure 4.7).

Membrane lipid peroxidation and its correlation to the RBC Hb - Hb-induced oxidative injury during RBC desiccation can compromise membrane integrity by lipid peroxidation and, consequently, contribute to hemolysis. This scenario was investigated by measuring TBARS in vacuum-dried RBCs of unmodified (oxyHb) and modified hemoglobins (carboxyHb, metHb) (Figure 4.8). TBARS levels were affected by two parameters: the sample's residual moisture content and the type of the RBC Hb (oxyHb, carboxyHb, metHb). High and medium residual moisture contents (15 ± 0.7 and 3.7 ± 0.2 g H_2O/g dry weight, respectively) were correlated with increased TBARS formation compared with lower residual moisture content (2.4 ± 0.4 g H_2O/g dry weight). This trend was observed in all three RBC groups.

Modifying the RBC hemoglobin altered the membrane susceptibility to lipid peroxidation in samples containing high and medium residual moisture contents (15 ± 0.7 g H_2O/g dry weight and 3.7 ± 0.2 g H_2O/g dry weight, respectively). TBARS levels in oxyHb RBCs were similar to or higher than that of carboxyHb RBCs, while metHb RBCs consistently contained lower TBARS levels than that of oxyHb RBCs and carboxyHb RBCs ($p=0.010$ and 0.003 at 15 ± 0.7 and 3.7 ± 0.2 g H_2O/g dry weight, respectively). The differences among the three RBC groups became insignificant ($p>0.05$) at low residual moisture contents (2.4 ± 0.4 g H_2O/g dry weight), where little TBARS formation was observed (Figure 4.8).

4.4 DISCUSSION

Correlation between Hb oxidation and ROS formation in vacuum-dried RBCs - Comparing ROS levels of reconstituted RBCs with unmodified (oxyHb) and modified (carboxyHb, metHb) hemoglobins revealed that ROS formation cannot be ascribed exclusively to Hb oxidation. This argument is based on the findings that ROS levels in carboxyHb and metHb RBCs (both have reduced capacity for Hb oxidation) were similar or higher than that of oxyHb RBCs (Figures 4.3 and 4.6). In the case of metHb RBCs, DCF fluorescence could have been affected by NOS generated by residual nitrite, which was used for the preparation of these cells. NOS, such as peroxynitrite ($ONOO^-$), can oxidize 2',7'-dichlorofluorescin to generate DCF fluorescence similar to that obtained by ROS [12]. However, NOS are likely to intensify oxidative stress and cellular injury, which contradicts our observations regarding the recovery of metHb RBCs (Figure 4.7). Future studies can benefit from using ROS-specific probes, such as polyethylene glycol-catalase (PEG-catalase) or polyethylene glycol-superoxide dismutase (PEG-SOD), which are less sensitive to NOS. Another aspect yet be resolved is the origin of these ROS. Possible sources are products of decomposed membrane lipids, loss of antioxidant activity, and sample reconstitution, which reintroduces oxygen that is available for redox reactions [13].

Reducing metHb formation during drying does not improve cell integrity - We predicted that blocking the Hb oxygen binding site would significantly reduce the rate of Hb oxidation during RBC desiccation and, consequently, improve cell integrity in carboxyHb RBCs. Such a scenario would have demonstrated that Hb oxidation is directly involved with cellular injury and hemolysis. We were able to decrease the rate of Hb oxidation during vacuum-drying of carboxyHb RBCs by about 50 % of that observed in

oxyHb RBCs (Figure 4.1). This action, however, failed to reduce the rates of intracellular ROS or hemolysis, which were higher than that of oxyHb RBCs (Figures 4.2 and 4.3). Choosing CO to block the Hb oxygen binding site is somewhat problematic due to possible dissociation of this ligand during vacuum-drying, storage and reconstitution. Our measurements showed that reconstituted carboxyHb RBCs contained about 75 % carboxyHb in contrast to freshly treated cells, which contained 90 %. CO dissociation releases the blockage from the heme iron and renders it available for redox reactions. Moreover, the presence of free CO within the RBC can be toxic and may have contributed to cellular damage.

Similar studies of carboxyHb RBCs have shown that blockage of the Hb oxygen binding site renders these cells more susceptible to membrane damage and hemolysis. Incubation of normal and sickle carboxyHb RBCs in the presence of activated neutrophils resulted in lipid peroxidation levels, which were significantly higher than that of oxyHb RBCs [14]. Similar observations have been described by Trotta *et al*, [15] who studied the reactions of t-BuOOH with RBCs. Claster *et al*. [14] suggested that hemoglobins are capable of free radical scavenging during oxidative stress in a mechanism that involves cycling between the hemoglobin's oxy and met states. Inhibiting this cycle by CO treatment may result in increased formation of intracellular ROS and, subsequently, oxidative injury. This idea is in agreement with our observation regarding the high levels of intracellular ROS monitored in vacuum-dried carboxyHb RBCs (Figure 4.3).

Our investigation of carboxyHb RBCs susceptibility to oxidation confirms that blocking the Hb oxygen binding site significantly reduces ROS formation upon H_2O_2 attack (Figure 4.4). Conversely, carboxyHb RBC susceptibility to t-BuOOH is similar to that of oxyHb RBCs. My interpretation of this data is that oxidative injury in desiccated

RBCs is mediated by lipid hydroperoxides, rather than H_2O_2. This observation is based on the fact that ROS levels in reconstituted carboxyHb RBCs are similar or higher than that of oxyHb RBCs (Figure 4.3), but not lower, as would have been expected in the case of H_2O_2-mediated injury (Figure 4.4). Moreover, H_2O_2 has been shown to intensify lipid peroxidation in the presence of metHb [16, 17], whereas we show that reconstituted metHb RBCs contain consistently lower levels of TBARS than that of carboxyHb and oxyHb RBCs (Figure 4.8).

Intracellular metHb is correlated with reduced cellular injury post reconstitution - Vacuum-drying of metHb RBCs has clarified the putative role that Hb plays in mediating oxidative injury during RBC desiccation. We predicted that high levels of intracellular metHb (95 %) would promote oxidative injury and, consequently, hemolysis. Our prediction is based on previous observations, which discuss the susceptibility of metHb to oxidative denaturation through the formation of irreversible hemichromes [18], or through its reactions with H_2O_2 [17]. Surprisingly, reconstituted metHb RBCs of medium residual moisture contents (around 5.5 to 3.5 g H_2O/g dry weight; Figure 4.7) exhibited lower hemolysis levels than that of oxyHb and carboxyHb RBCs.

One explanation for the improved cell integrity of reconstituted metHb RBCs can be found in the low levels of TBARS observed in our study (Figure 4.8). Resistance of metHb RBCs to membrane lipid peroxidation has been documented during incubations with activated neutrophils [14] or in response to t-BuOOH oxidation [15]. In both cases, metHb RBCs exhibited lower levels of lipid peroxidation than that of carboxyHb and oxyHb RBCs. Sztiller *et al*, [19] have demonstrated that the incubation of metHb with RBC membranes or intact RBCs does not initiate lipid peroxidation. These beneficial effects of metHb are likely to occur as long as this molecule is stable, as metHb

degradation releases toxic substances, which would exacerbate the oxidative injury. We did not detect hemichromes in our reconstituted metHb RBCs, which suggests that desiccation under the tested conditions did not compromise metHb integrity.

Hb-mediated hemolysis during desiccation is water dependent - Hb oxidation is one of multiple factors capable of promoting hemolysis during RBC desiccation and dry storage. This study indicates that the incidence of Hb-mediated cellular injury is higher in stored RBC samples of medium residual moisture contents (around 5.5 to 3.5 g H_2O/g dry weight) than in more fully desiccated samples ($\leq$ 2 g H_2O/g dry weight). Medium moisture content is the range where modifications in the Hb ligand state significantly affected cell integrity (Figure 4.7) and membrane damage due to lipid peroxidation (Figure 4.8). These observations imply that water plays a critical role during Hb-mediated hemolysis.

In desiccation-tolerant organisms, different stages of water loss are characterized by different mechanisms of damage, which require modifications of survival strategies [20]. Protein unfolding, impaired enzyme activity and membrane perturbations can take place at early stages of dehydration. Such a scenario can lead to compromised metabolic activity at residual moisture contents that are sufficient for chemical reactions. In reference to partially dehydrated RBCs, changes in the activity of vital antioxidants, such as metHb reductase, glutathione peroxidase and catalase, may account for the drastic increases in Hb oxidation and membrane lipid peroxidation.

Cellular injury in RBC samples stored under lower residual moisture contents (2.4 $\pm$ 0.4 g H_2O/g dry weight) seems to be triggered primarily by physical and mechanical stress, such as osmotic shock and, to a lesser extent, by the Hb. This is seen

by the insignificant differences in hemolysis levels between RBC samples that contained different levels of metHb (10 % in oxyHb RBCs versus 95 % in metHb RBCs; Figure 4.7). Similarly, the differences in TBARS levels among the three RBC groups become insignificant at this range of residual moisture content (Figure 4.8). Moreover, the low levels of TBARS observed in all RBC groups suggest that lipid peroxidation did not play a major role in mediating membrane damage and hemolysis.

Data presented in Chapters 3 and 4 provide a new mechanistic understanding of the correlation between desiccation and oxidative injury in RBCs. It defines the residual moisture contents where Hb redox reactions can affect cell integrity, and demonstrates how pharmacological intervention can mitigate these effects. The nature of these redox reactions is not clear and may involve the oxidation of the heme iron to the highly reactive ferryl state (Fe^{4+}), which yields ferrylHb (see section 1.2.3 of Chapter 1 for review). The latter is a strong oxidizing agent capable of increasing intracellular levels of metHb through its reactions with oxyHb, as well as promoting heme degradation in the presence of peroxides [21, 22]. Further studies should verify whether ferrylHb is involved in mediating cellular damage during RBC desiccation. The next chapter expands our understanding of the RBC oxidative injury by examining the role glucose and glutathione play during desiccation stress.

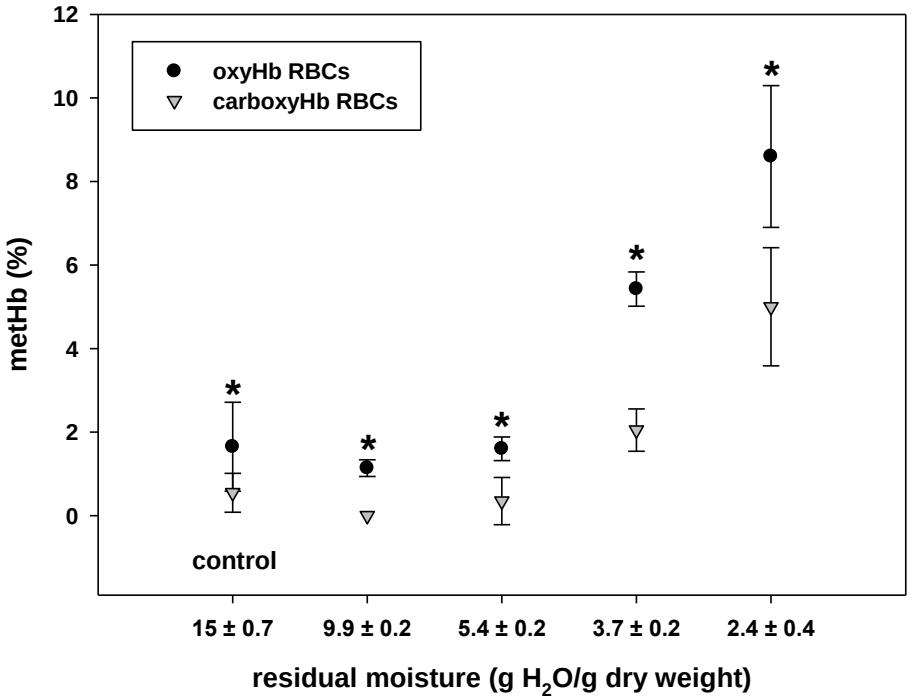

Figure 4.1. Vacuum-drying of carboxyHb RBCs: Hb oxidation. Carbon monoxide-treated RBCs (carboxyHb RBCs) and untreated RBCs (oxyHb RBCs) were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Control RBCs (15 ± 0.7 g H_2O/g dry weight) were incubated for 7 days without being exposed to vacuum-drying. Rate of Hb oxidation is represented as percent metHb. Asterisk symbols (*) denote significant differences between the two RBC groups. Error bars represent standard deviation. Significant differences ($p<0.0001$) between the two RBC groups were observed in all tested ranges of residual moisture contents (n=4-12).

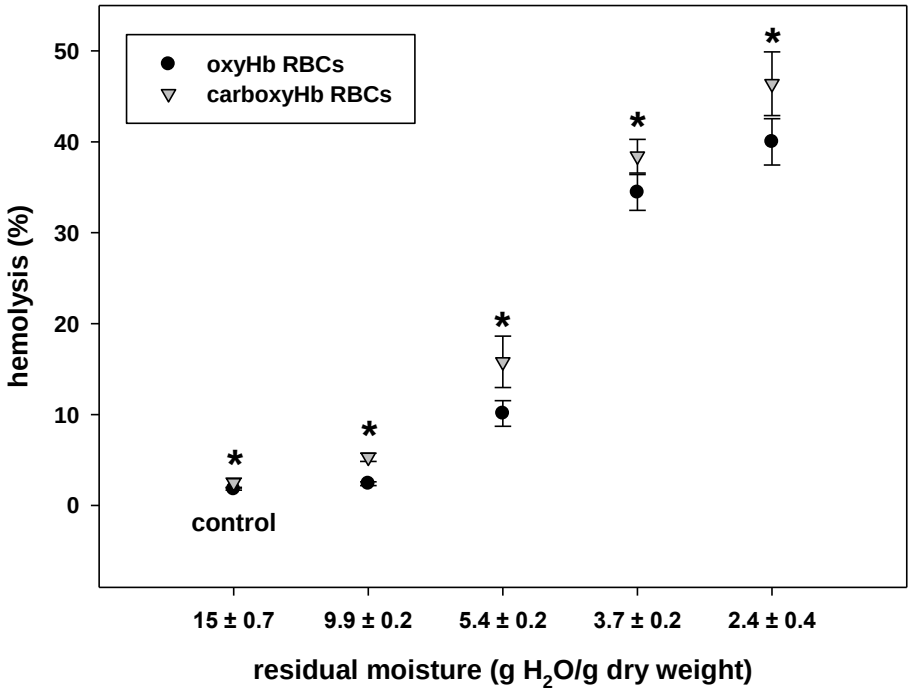

Figure 4.2. Vacuum-drying of carboxyHb RBCs: Hemolysis. Carbon monoxide-treated RBCs (carboxyHb RBCs) and untreated RBCs (oxyHb RBCs) were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Control RBCs (15 ± 0.7 g H_2O/g dry weight) were incubated for 7 days without being exposed to vacuum-drying. Cell integrity is represented as percent hemolysis. Asterisk symbols (*) denote significant differences between the two RBC groups. Error bars represent standard deviation. Significant differences ($p < 0.05$) between the RBC groups were observed in all tested ranges of residual moisture contents (n=4-12).

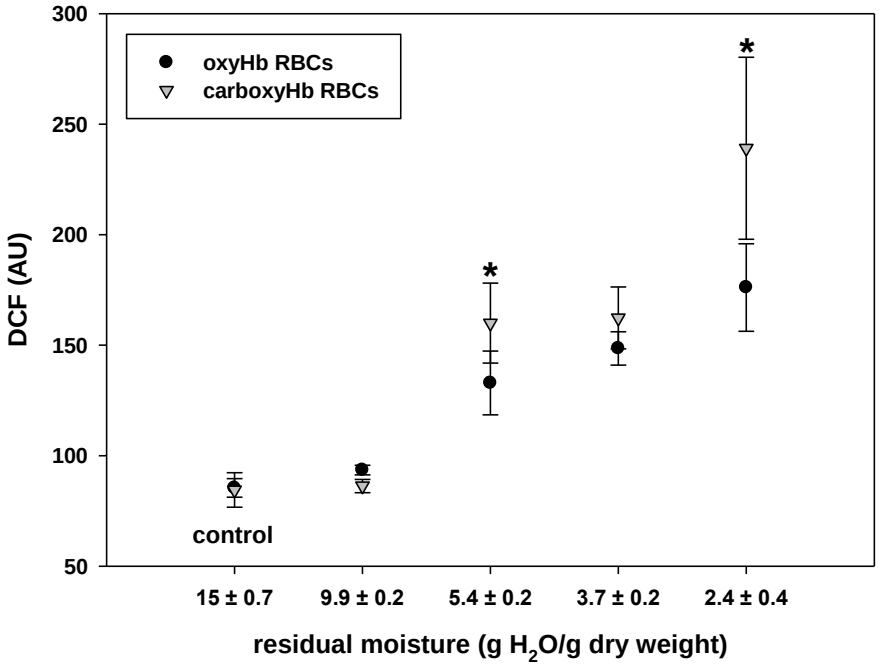

Figure 4.3. Vacuum-drying of carboxyHb RBCs: ROS formation. Carbon monoxide-treated RBCs (carboxyHb RBCs) and untreated RBCs (oxyHb RBCs) were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Control RBCs (15 ± 0.7 g H_2O/g dry weight) were incubated for 7 days without being exposed to vacuum-drying. ROS formation is represented as DCF fluorescence (arbitrary units). Asterisk symbols (*) denote significant differences between the two RBC groups. Error bars represent standard deviation. Significant differences between the two RBC groups were observed at 5.4 ± 0.2 and 2.4 ± 0.4 g H_2O/g dry weight ($p=0.009$ and 0.043, respectively) (n=4-12).

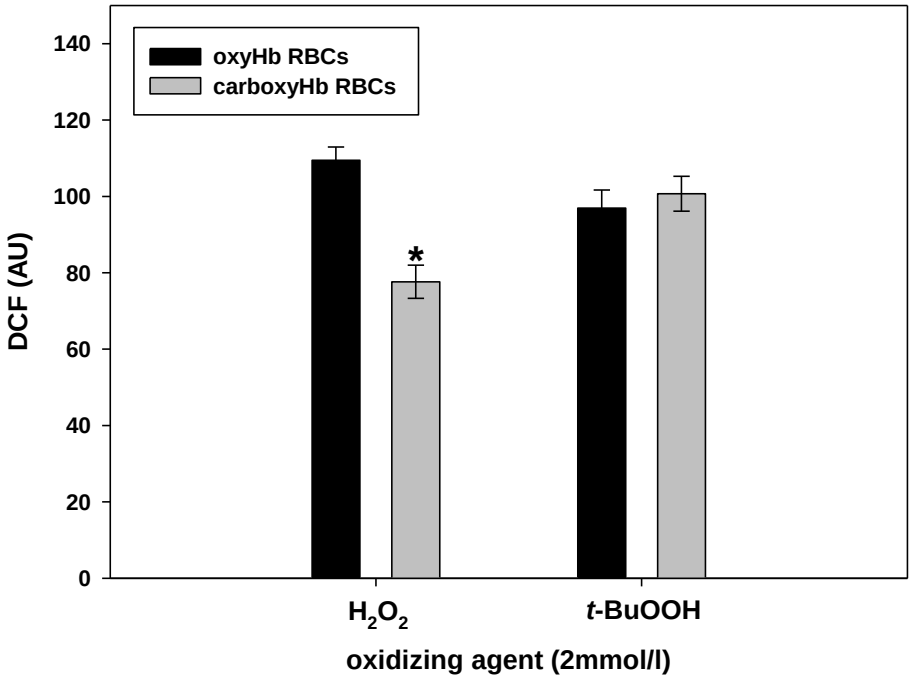

Figure 4.4. Oxidative challenge of carboxyHb RBCs. ROS formation in oxyHb and carboxyHb RBCs in response to oxidative challenge by H_2O_2 [2 mmol/l] or t-BuOOH [2 mmol/l]. A significant difference (p= 0.009) between the two red blood cell groups was observed in response to H_2O_2 treatment (n=5). Asterisk symbol (*) denotes a significant difference between the two red blood cell groups in response to H_2O_2 oxidation. Error bars represent standard deviation.

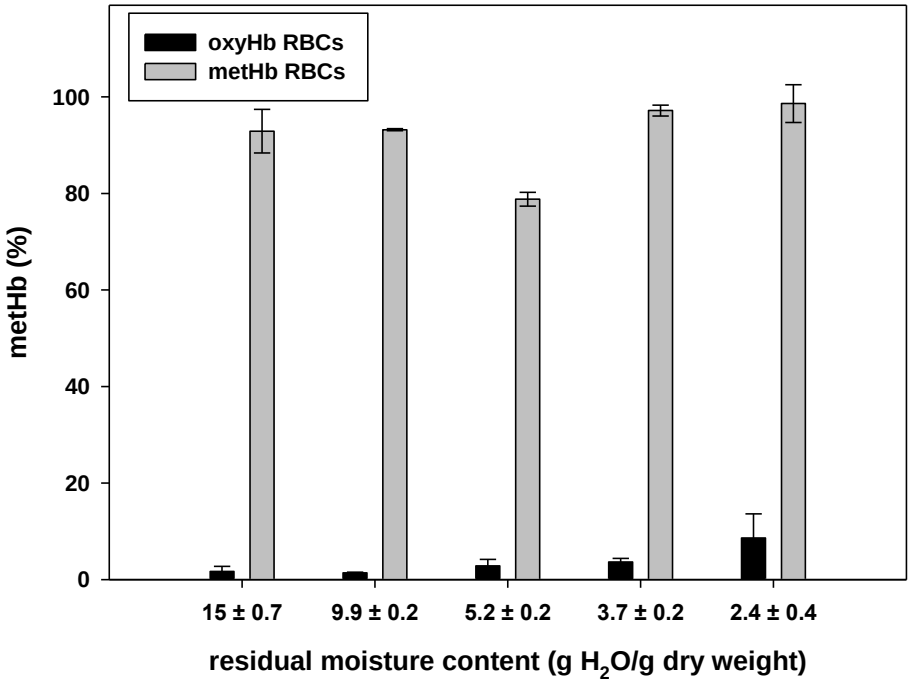

Figure 4.5. Vacuum-drying of metHb RBCs: Hb oxidation. MetHb RBCs and oxyHb RBCs were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Control RBCs (15 ± 0.7 g H_2O/g dry weight) were incubated for 7 days without being exposed to vacuum-drying. Oxidized Hb is represented as percent metHb. Error bars represent standard deviation (n=4-12).

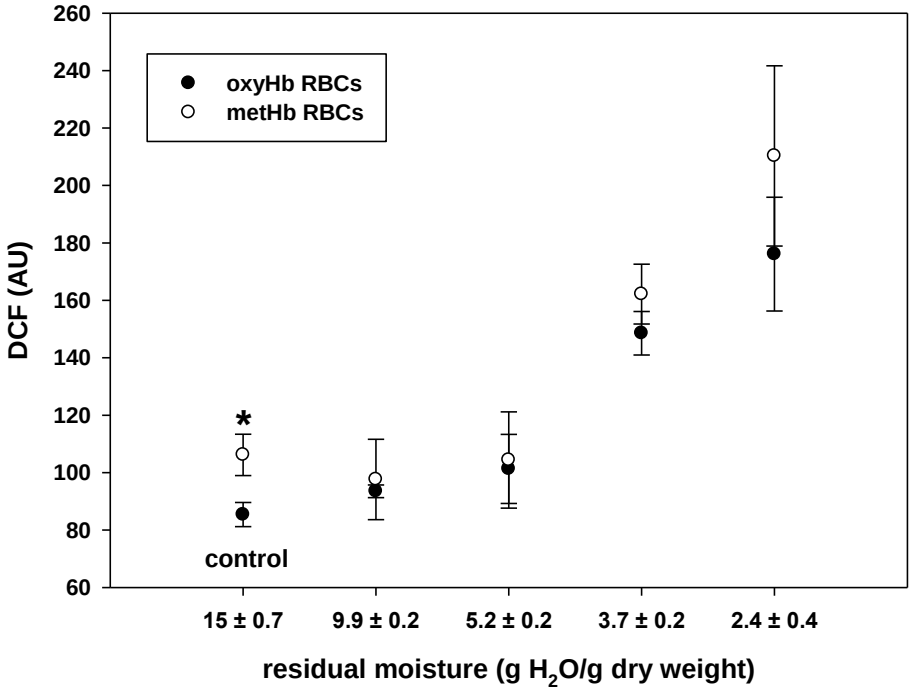

Figure 4.6. Vacuum-drying of metHb RBCs: ROS formation. MetHb RBCs and oxyHb RBCs were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Control RBCs (15 ± 0.7 g H_2O/g dry weight) were incubated for 7 days without being exposed to vacuum-drying. ROS formation is represented as DCF fluorescence (arbitrary units). Asterisk symbols (*) denote significant differences between the two RBC groups. Error bars represent standard deviation. Significant differences ($p<0.0001$) between the two RBC groups were observed in control samples (n=4-12).

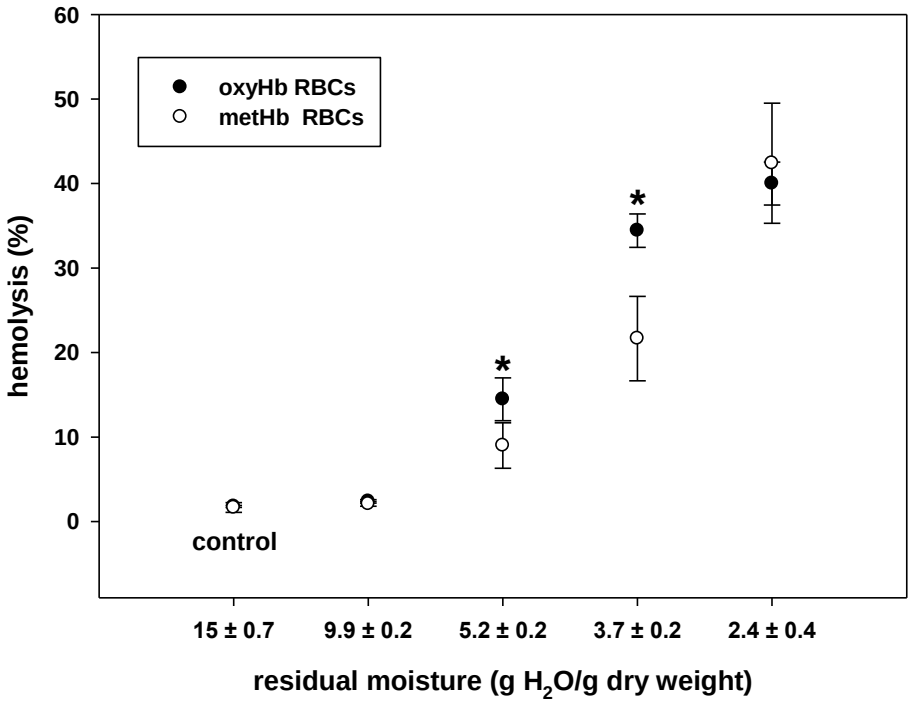

Figure 4.7. Vacuum-drying of metHb RBCs: Hemolysis. MetHb RBCs and oxyHb RBCs were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Control RBCs (15 ± 0.7 g H_2O/g dry weight) were incubated for 7 days without being exposed to vacuum-drying. Cell integrity is represented as percent hemolysis. Asterisk symbols (*) denote significant differences between the two RBC groups. Error bars represent standard deviation. Significant differences between the two RBC groups were observed at 5.2 ± 0.2 and 3.7 ± 0.2 g H_2O/g dry weight (p= 0.01 and 0.021, respectively) (n=4-12).

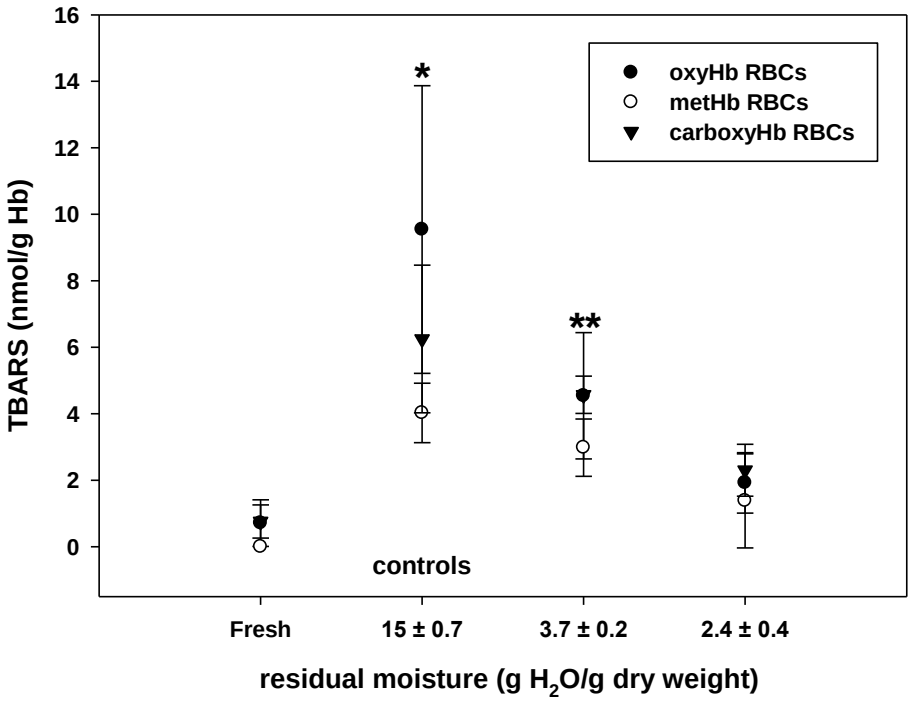

Figure 4.8. Membrane lipid peroxidation in vacuum-dried RBCs. RBCs with unmodified (oxyHb RBCs) or modified hemoglobins (carboxyHb, metHb RBCs) were vacuum-dried to defined residual moisture contents and stored at 25 °C for a total period of 7 days. Lipid peroxidation in reconstituted RBCs is represented by formation of TBARS (nmol/g Hb). Significant differences in TBARS levels among the three RBC groups were obtained from one way ANOVA. A single asterisk symbol (*) denotes significant differences ($p= 0.010$) at 15 ± 0.7 g H_2O/g dry weight. Double asterisk symbol (**) denote significant differences ($p= 0.003$) at 3.7 ± 0.2 g H_2O/g dry weight. Error bars represent standard deviation (n= 4-12).

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CHAPTER 5: Glucose, glutathione and the RBC resistance to desiccation stress

5.1 INTRODUCTION

The data presented so far (chapters 3 and 4) is based on an experimental design, which did not include glucose in the drying buffer. Glucose, a reducing sugar, can significantly affect cellular recovery during *ex vivo* preservation of RBCs and under desiccation stress. At physiological concentrations (3.9 to 5.8 mmol/L), glucose can prevent cellular injury by sustaining certain metabolic pathways such as Embden-Meyerhof, in which adenosine triphosphate (ATP) is produced, or the hexose-mono-phosphate shunt (HMPS) that is necessary for synthesis of nicotinamide adenine dinucleotide phosphate (NADPH) [1]. The latter is vital for regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) (Equation 5.1) in RBCs and other cell types [2].

Reduction of GSSG to GSH

$$\mathbf{GSSG + NADPH + H^{+} \xrightarrow{\text{glutathione reductase}} 2GSH + NADP^{+}} \quad \mathbf{(5.1)}$$

Exposing RBCs to glucose at concentrations exceeding physiological levels can be deleterious due to non-enzymatic glycosylation of cytosolic proteins and formation of cytotoxic advanced glycation end products (AGE) [3]. Non-enzymatic glycation of Hb has been correlated with ROS formation and membrane lipid peroxidation in RBCs of diabetes patients [4]. Intracellular AGE formation has been documented during hypothermic storage of RBC units in adenine-dextrose-sodium chloride-mannitol (ADSOL) solution [3]. The high concentration of glucose in this storage solution (122

mmol/L) [5] is potentially toxic and can contribute to membrane damage commonly observed with prolonged hypothermic storage of RBCs.

Glucose has been extensively used as an intracellular protectant against osmotic stress in lyophilized RBCs [6-9]. The rationale is that glucose helps maintaining osmotic balance between the intracellular and extracellular milieu, especially in the presence of membrane-impermeable excipients (i.e. disaccharides, polyvinylpyrrolidone) [10]. The beneficial effects of glucose for cryopreservation applications are questionable, because the concentrations required for improved cell integrity are extremely high (about 20-30 % wt/vol). Most cryopreservation studies have been oblivious to glucose toxicity, except for one that linked glucose overload with membrane damage in terms of phosphatidylserine exposure [7].

In the present study, vacuum-drying and storage of RBCs have been carried out in the absence of glucose, under conditions that would allow a certain degree of metabolic activity (25 °C). Glucose starvation during desiccation stress can compromise the RBC HMPS metabolic pathway and, consequently, exhaust cellular mechanisms necessary for detoxification of ROS. In regard to the general hypothesis, glucose starvation could have exacerbated the rates of Hb oxidation and ROS formation observed in vacuum-dried RBCs. The first aim of this chapter is to investigate the correlation between glucose and cellular injury in vacuum-dried RBCs.

The second aim of this chapter deals with glutathione depletion as a possible consequence of glucose starvation. Glutathione (γ-L-glutamyl-L-cysteinylglycine), a water-soluble antioxidant necessary for detoxification of hydrogen peroxide (Equation 5.2) [2], has been shown to play a critical role in enhancing desiccation tolerance of

various anhydrobiots including lichen, orthodox seeds and resurrection plants [11]. In yeast cells, knock-out of glutathione genes drastically exacerbated the rate of oxidative injury and cellular death following desiccation stress [12].

Elimination of hydrogen peroxide by GSH

$$H_2O_2 \xrightarrow{\text{2GSH to GSSG}} 2H_2O \qquad (5.2)$$

Studies of the RBC hypothermic storage lesion suggested that the exhaustion of the glutathione system (GSH, glutathione reductase and glutathione peroxidase) can significantly contribute to cellular injury during prolonged hypothermic storage [13]. Dumaswala *et al*. demonstrated that hypothermically stored RBCs suffered a time-dependent decrease of about 30 % in GSH concentration, which was correlated with oxidative injury to membrane lipids and proteins. A subsequent study showed that glutathione loading into RBCs can reduce the extent of oxidative injury in hypothermically stored RBCs [5, 13].

On the other hand, studies of hemoglobin pathologies have shown that glutathione can act as a prooxidant in the presence of free heme [14]. Unstable hemoglobins, such as free α-hemoglobin chains in thalassemic RBCs, tend to release their heme moiety in response to autoxidation pathways [15]. GSH has been shown to catalyse the destruction of free and membrane-bound heme in a reaction, which promotes oxidative injury through the formation of thiyl radicals ($GS^{\cdot}$) and the release of iron atoms [14]. Interestingly, GSH depletion in thalassemic RBCs rendered these cells more resistant to oxidative injury [15]. This chapter addresses the role glutathione plays during RBC desiccation by examining the consequences of glutathione depletion on the recovery of vacuum-dried RBCs.

5.2 MATERIALS AND METHODS

Reagents - All reagents are of analytical grade. Dimethyl sulfoxide (Me_2SO) was purchased from Fisher Chemical (Fair Lawn, NJ, USA). Sodium chloride, HEPES, glucose, DL-buthionine-(S,R)-sulfoximine (BSO), tert-Butyl hydroperoxide solution (t-BuOOH; 70 % aqueous solution), hydrogen peroxide, 2',7'-dichlorofluorescin diacetate (DCFH-DA), and Drabkin's reagent components (Triton x-100, potassium dihydrogen phosphate, potassium cyanide and potassium ferricyanide (III)) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Preparation of human RBC suspensions - Leukocyte-reduced packed RBC units stored in saline-adenine-glucose-mannitol (SAGM) were provided by Canadian Blood Services. These units were produced from whole blood collections obtained from healthy donors. A units' age varied from 3 to 14 days (median = 7 days). The RBCs were washed twice (10 min, 3200 g, 4 °C) with HEPES buffer (20 mmol/L HEPES, 136 mmol/L sodium chloride, pH 7.4) to remove the additive solution (SAGM), the supernatant was discarded, and the pellet was suspended in the same buffer. For the glucose study, washed RBCs were suspended with HEPES-glucose buffer containing 20 mmol/L HEPES, 136 mmol/L sodium chloride, and 5 mmol/L glucose (pH 7.4). Glucose was added at 5 mmol/L to mimic physiological levels. The hematocrit (HCT) of each RBC suspension was determined by micro-hematocrit centrifugation (Hettich Haematokrit) and averaged 15 % ± 2 %.

Glutathione depletion - Glutathione depletion was achieved by pre-treatment of RBC suspensions with BSO, a specific inhibitor of γ-glutamylcysteine synthetase that is

necessary for GSH synthesis [16]. A 200 mmol/L BSO stock solution in HEPES buffer was diluted with RBC suspensions to a final concentration of 5 mmol/L. Untreated and BSO-treated RBCs were then incubated for 22 h at 37 °C. After the incubation period, the RBC suspensions were assayed for percent hemolysis and percent metHb, and vacuum-dried as described below.

Vacuum drying - Untreated, glucose-treated, or BSO-treated RBC samples (2 mL) were placed in Falcon® 25 cm^2 non-treated cell culture flasks (Becton Dickinson and Company, NJ, USA). Flask weight (g) was determined before and after the addition of the RBC sample by using a digital balance (PG603-S, Mettler Toledo, Switzerland). Flasks containing RBC samples were placed in a vacuum oven (Fisher Isotemp 282A, Fisher Scientific Canada) and vacuum dried at 25 °C under low pressure (55 ± 5 Torr) for time lengths ranging from 70 h to 76 h. At the end of each drying cycle, samples were weighed, sealed (flask's cap closed and Parafilm®) and stored at 25 °C (atmospheric pressure) for 4 days. At the end of the incubation period, control and vacuum-dried RBC samples were weighed to determine the residual moisture content (g H_2O/ g dry weight). Sample water loss during storage was negligible (≤ 2-3 % of total mass). Vacuum-dried samples were rehydrated in one step by adding HEPES buffer (37 °C). The quantity (mL) of the added buffer corresponded to the amount of water required to obtain the original volume (2 mL).

Determination of residual moisture - The residual moisture content (g H_2O/ g dry weight) of vacuum dried RBC samples was determined by gravimetric analysis [17], similar to the procedure described by Ma *et al*, [18]. Sample dry weight (g) was measured in duplicates by baking vacuum dried samples for 48 h at 80 °C. Sample water weight (g H_2O) was obtained by measuring the difference between the sample weight and the

averaged dry weight of at least two identical samples. The residual moisture content is represented as g H_2O/ g dry weight. Prior to drying, RBC samples contained 15 ± 1.5 g H_2O/ g dry weight. Vacuum dried samples containing less than 1 g H_2O/ g dry weight were considered to be fully desiccated.

Determination of percent hemolysis - Percent hemolysis was determined by comparing the supernatant Hb to total Hb concentrations, using Drabkin's method [19, 20]. The HCT of each sample was measured before assaying using the micro-hematocrit centrifuge. Percent hemolysis was calculated from the following equation:

$$\%\ \text{hemolysis} = \frac{(100 - \text{HCT})\text{Hb}_\text{s}}{\text{Hb}_\text{t}}$$

Determination of hemoglobin oxidation - The percentage of oxidized hemoglobin (methemoglobin) in RBC suspensions was determined by spectrophotometry using the millimolar extinction coefficients of the different Hb types (oxyhemoglobin, methemoglobin, hemichromes) at pH 7.4 [21]. Briefly, RBC lysates were scanned from 500 nm to 700 nm while recording the absorbance values (OD) at 560 nm, 577 nm, 630 nm and 700 nm. The concentrations (mmol/L) of the different Hb types were then calculated as follows:

$$[\text{OxyHb}] = 29.8(OD_{577}\text{-}OD_{700})\text{-}9.8(OD_{630}\text{-}OD_{700})\text{-}22.2(OD_{560}\text{-}OD_{700})$$

$$[\text{MetHb}] = 7(OD_{577}\text{-}OD_{700}) + 76.8(OD_{630}\text{-}OD_{700})\text{-}13.8(OD_{560}\text{-}OD_{700})$$

$$[\text{Hemichromes}] = -33.2(OD_{577}\text{-}OD_{700})\text{-}36(OD_{630}\text{-}OD_{700}) + 58.2(OD_{560}\text{-}OD_{700})$$

Determination of intracellular reactive oxygen species - Formation of intracellular ROS was monitored by flow cytometry using the DCFH-DA probe. Control

and reconstituted RBCs were labeled with DCFH-DA by diluting a DCFH-DA stock solution (40 mmol/L dissolved in Me_2SO) with each RBC sample to a final concentration of 0.4 mmol/L. The RBCs were incubated for 15 min at 37 °C on a nutating mixer, washed (10 min, 1000g, 4 °C), diluted with HEPES buffer to a final concentration of $5x10^6$ cells/mL, and analyzed by flow cytometry.

Determination of heme degradation - Nagababu *et al*, demonstrated that the reaction of oxyHb with hydrogen peroxide results in heme degradation and the release of two fluorescence products, which can be monitored by spectrophotometry or flow cytometry [22, 23]. Conversely, the reaction of metHb with hydrogen peroxide does not produce autofluorescence [22]. Control and reconstituted RBCs were diluted with HEPES buffer to a final concentration of $5x10^6$ cells/mL and analysed by flow cytometry as described below. Positive controls were prepared by challenging RBCs with either hydrogen peroxide [2 mmol/L] or t-BuOOH [2 mmol/L]. The addition of each oxidizing agent was followed by 15 min incubation at 37 °C using mild agitation. Oxidized RBC samples were then washed, suspended with HEPES buffer, and analysed by flow cytometry. RBC oxidation by hydrogen peroxide was preceded by sodium azide treatment (1 mmol/L) to inhibit catalase activity.

Flow cytometric analyses - Unlabeled (heme degradation assay) and DCF-labeled RBCs were analysed on a flow cytometer (FACScan, BD Sciences, San Jose, CA, USA) equipped with an argon laser (488 nm). Acquisition and analysis were performed using CellQuest® software (BD Sciences). Forward scatter (FSC) was plotted against the side scatter (SSC) on a linear scale. A total of 10,000 events were collected from each sample and detected by the FL-1 channel using log amplification. The mean fluorescence intensity of the entire RBC population was obtained by CellQuest® software.

Statistical analysis - A nonparametric statistical analysis was performed using commercial software (SPSS, Lead Technologies, Charlotte, NC, USA). The differences between two independent groups were analysed using Mann-Whitney U test. Probabilities less than 0.05 were considered significant.

5.3 RESULTS

Vacuum-drying of glucose-treated RBCs - The consequences of adding glucose (5 mmol/L) to the drying buffer were studied on partially dehydrated RBC samples (5.8 ± 0.3 g H_2O/g dry weight) and relatively dry RBC samples (2.8 ± 0.5 g H_2O/g dry weight). The presence of glucose in the drying media significantly affected cellular recovery in partially dehydrated RBCs samples. MetHb levels in glucose-treated RBCs were about twice of that of untreated RBCs at both residual moisture contents (Figures 5.1 and 5.4); however, at 5.8 ± 0.3 g H_2O/g dry weight, the absolute values of percent metHb in untreated and glucose-treated RBCs (< 1 % and 1.4 ± 0.2 %, respectively) were within the physiological range (0.5 % to 3 %).

Glucose treatment effectively reduced ROS formation and hemolysis levels in partially dehydrated RBC samples. ROS levels in glucose-treated RBCs were about 30 % lower than that of untreated RBCs (p=0.002, Figure 5.2). The difference in cell integrity between the two RBC groups was even more dramatic, as hemolysis levels in glucose-treated RBCs were about 25 % of that of untreated RBCs (p=0.002, Figure 5.3).

Contrary to partially dehydrated RBC samples, glucose had no beneficial effects on the recovery of relatively dry RBC samples. MetHb levels in glucose-treated RBCs measured about 10 % versus untreated RBCs, which contained about 5 % (Figure 5.4). Similar levels of ROS were observed between the two RBC groups (Figure 5.5).

Conversely, hemolysis levels were about 10 % higher in glucose-treated RBCs compared with untreated RBCs (figure 5.6).

Vacuum-drying of BSO-treated RBCs - Pre-treatment of RBCs with BSO (22 h, 37 °C) did not induce cellular injury in terms of hemolysis, which levels were similar to that of untreated RBCs and measured less than 1 % (data not shown). MetHb levels in both RBC groups were about 1.5 %. Following BSO treatment, RBCs were vacuum-dried to a medium range of residual moisture content (5.0 ± 0.5 g H_2O/g dry weight). This range was chosen for its efficiency of studying the effects of pharmacological treatments on vacuum-dried RBCs (see Chapter 4).

Under the tested conditions, vacuum-drying of BSO-treated RBCs had no significant effects on the RBC oxidative state in terms of Hb oxidation and ROS formation. MetHb levels in untreated and BSO-treated RBCs were similar ($p=0.059$) and measured about 10 % (Figure 5.7). Correspondingly, no significant differences ($p=0.462$) were observed in the ROS levels of untreated and BSO-treated RBCs (Figure 5.8). Conversely, a significant ($p=0.006$) decrease of 34 % in hemolysis levels was observed in BSO-treated RBCs compared with untreated RBCs (figure 5.9).

The difference in hemolysis levels between the two RBC groups (Figure 5.9) was further characterized by monitoring heme degradation products in reconstituted RBCs. As discussed in the introduction section, GSH can act as a prooxidant in the presence of free heme and, consequently, promote hemolysis. Such a scenario can explain the higher levels of hemolysis observed with untreated RBCs. Challenging RBCs with hydrogen peroxide or t-BuOOH served as a positive control for monitoring changes in intracellular autofluorescence (Figure 5.10). Both oxidizing agents caused a dose response increase in

intracellular autofluorescence, with hydrogen peroxide being more potent at the tested concentrations (0.5, 1, and 2 mmol/L).

Vacuum-drying caused a significant ($p<0.001$) increase (9 %) in cellular autofluorescence of BSO-treated and untreated RBCs (Figure 5.11). Autofluorescence levels in fresh and reconstituted BSO-treated RBCs were similar to that of untreated RBCs. Fresh samples from both RBCs groups had mean fluorescence intensity of 1.05 AU while reconstituted samples averaged 1.14 AU (Figure 5.11).

5.4 DISCUSSION

Correlation between glucose and cellular injury in vacuum-dried RBCs - This study examined the correlation between glucose and cellular injury in vacuum-dried RBCs. The premise was that the drying and storage conditions used for this study (25 °C) do not suspend metabolic activity and, thus, glucose starvation could have contributed to desiccation-induced oxidative injury. In this study design, glucose was added to the drying media at a physiological concentration (5 mmol/L) to avoid cellular injury that is correlated with higher concentrations of glucose [24].

Glucose ability to reduce cellular injury during RBC desiccation appears to be water dependent. At medium levels of residual moisture content (5.8 ± 0.3 g H_2O/g dry weight), glucose significantly improved cellular recovery compared with untreated RBCs (Figures 5.2 and 5.3). Conversely, glucose failed to protect RBCs from cellular injury at lower levels of residual moisture contents (2.8 ± 0.5 g H_2O/g dry weight; Figures 5.4, 5.5 and 5.6).

The low levels of hemolysis (Figure 5.3) observed with glucose-treated RBCs at 5.8 ± 0.3 g H_2O/g dry weight can be explained by the ability of the RBC to sustain

metabolic activity at this range of residual moisture. Glucose catabolism through the Embden-Meyerhof pathway would provide the RBC with ATP, which is necessary for the maintenance of various cell functions such as electrolyte balance and antioxidant activity [25]. ATP depletion during hypothermic storage of RBCs has been correlated with membrane injury and changes in rheological characteristics in response to lipid loss, microvesiculation and reduced surface area to volume ratio [26]. As discussed in the introduction section, glucose catabolism through the HMFS pathway is vital for maintenance of the RBC reducing power. This can explain the lower levels of ROS observed with glucose-treated RBCs in comparison to untreated RBCs (Figure 5.2).

Interestingly, the lower levels of ROS monitored in glucose-treated RBCs of medium residual moisture contents (Figure 5.2) were not correlated with reduced levels of Hb oxidation (Figure 5.1). On the contrary, glucose-treated RBCs contained higher levels of metHb than that of untreated RBCs. This finding brings back the question regarding the source of desiccation-induced ROS. Chapter 4 concluded that ROS formation in desiccated RBCs cannot be ascribed exclusively to Hb oxidation because similar levels of ROS were monitored with RBCs, which contained different levels of metHb. This chapter supports this idea by showing an inverse relationship between Hb oxidation and ROS formation in glucose-treated RBCs (Figures 5.1 and 5.2).

Another aspect, which is in agreement with previous findings, is the efficacy of pharmacological treatments at different ranges of residual moisture contents. Similarly to observations with carboxyHb and metHb RBCs (Chapter 4), the effects of glucose treatment were more pronounced in partially dehydrated RBC samples than in relatively dried samples. This finding supports the argument that susceptibility to oxidative injury changes at different levels of dehydration. In the case of glucose, beneficial effects at 5.8

± 0.3 g H_2O/g dry weight turn into rather deleterious effects at 2.8 ± 0.5 g H_2O/g dry weight.

One observation that is common to both ranges of residual moisture contents is that glucose treatment promotes Hb oxidation (Figures 5.1 and 5.4). As demonstrated with the case of metHb RBCs (Chapter 4), high levels of intracellular metHb are not necessarily correlated with cellular injury; on the contrary, metHb RBCs demonstrated higher levels of cell integrity at medium levels of residual moisture content. In the case of glucose-treated RBCs, the nature of the reactions between glucose and hemoglobins is not clear. The possibility of AGE formation seems unlikely because several studies of RBC hyperglycemia have shown that RBC incubation with 5 mmol/L glucose does not induce AGE [24, 27, 28]. In fact, this concentration of glucose is often used as a control in the studies of hyperglycemic RBCs.

The scenario of hemoglobin glycosylation in vacuum-dried RBCs cannot be ruled out completely. As discussed in Chapter 1, drying increases the concentration of intracellular solutes. In the case of glucose-treated RBCs, vacuum-drying to low residual moisture contents can theoretically increase the concentration of glucose to levels that could promote cellular injury in response to non-enzymatic glycation of lipids and proteins including hemoglobins. However, hemoglobin glycosylation cannot explain the high levels of metHb observed in vacuum-dried glucose-treated RBCs (Figure 5.4) because glucose binds to valine residues of the globin chains rather than to the heme pocket [29]. In other words, this reaction does not require transition metals and should not affect the oxidative state of the heme iron.

A different reaction, which can oxidize the heme iron, is glucose autoxidation. The latter can reduce molecular oxygen under physiological conditions and yield α-ketoaldehydes, hydrogen peroxide and other free radical intermediates. Moreover, this reaction is catalyzed by transition metals [28]. This can explain the 2-fold increase in metHb levels monitored in glucose-treated RBCs of low residual moisture content (Figure 5.4). Monitoring for glucose autoxidation products, such as α-ketoaldehydes, in desiccated RBCs would help clarifying the role glucose plays in mediating cellular injury at low residual moisture contents.

BSO-treatment is correlated with improved cell integrity post reconstitution - Glutathione has been shown to play a critical role in enhancing desiccation-tolerance in a variety of organisms. Espindola *et al*, argued that enzymatic antioxidant systems cannot operate in the absence of water and, thereby, become irrelevant in the dried state. Instead, molecular antioxidants, such as GSH, provide the main defense mechanism against desiccation-induced oxidative injury [12]. Based on evidences from desiccation-tolerant organisms and hypothermic storage of RBCs, glutathione depletion was expected to exacerbate the extent of desiccation-induced oxidative injury and compromise cellular recovery.

Contrary to this prediction, RBC BSO treatment significantly ($p=0.006$) improved cell integrity following vacuum-drying, storage and reconstitution (Figure 5.9). BSO, a glutathione depleting agent, has been shown to increase intracellular ROS levels and, consequently, promote apoptosis in various cell lines including neuroblastoma [16]. In this study, BSO did not seem to act as a prooxidant as seen by insignificant differences in metHb and ROS levels of BSO-treated and untreated RBCs (Figures 5.7 and 5.8, respectively). This observation suggests that under the tested conditions, glutathione may

not play a major role in protecting the RBCs against oxidative damage during desiccation.

The observation of improved cell integrity in vacuum-dried BSO-treated RBCs (Figure 5.9) requires further investigation. One scenario where GSH depletion in RBCs can improve cell integrity can be found in the works of hemoglobin disorders. RBCs from thalassemic patients contain high levels of free heme due to degradation of unpaired hemoglobins [15, 30]. As mentioned in the introduction, GSH can act as a prooxidant in the presence of free heme and, thus, promote hemolysis. Breakage of the heme ring is reflected by increases of cellular autofluorescence, which can be monitored by flow cytometry [23]. RBC treatments with hydrogen peroxide or t-BuOOH (Figure 5.10) confirmed previous observations regarding the increase of cellular autofluorescence in response to oxidation.

Vacuum-drying of BSO-treated and untreated RBCs was also correlated with a significant increase ($p<0.001$) in intracellular autofluorescence (Figure 5.11). The rate of this increase (9 %) was relatively modest compared with levels monitored in positive controls (Figure 5.10). Whether the increase in autofluorescence is sufficient to execute the differences in hemolysis levels between the two RBC groups (Figure 5.9) is yet to be resolved. Clarification of this issue can be done by monitoring vacuum-dried RBCs for intracellular levels of GSH and GGSG, free and membrane-bound heme, and oxidized lipids.

The overall levels of metHb (~ 10 %) and hemolysis (~ 20 %) of vacuum-dried untreated RBCs (Figures 5.7 and 5.9) were relatively higher than expected at the range of about 5 g H_2O/ g dry weight. Similar experiments with untreated RBCs (oxyHb RBCs,

which were vacuum-dried to 5.2 ± 0.2 g H_2O/ g dry weight; Figures 4.5 and 4.7) resulted in lower levels of metHb (~ 3 %) and hemolysis (~ 15 %). The relatively higher levels of metHb and hemolysis observed with untreated RBCs of the BSO study can be related to the prolonged incubation period (22 h, 37 °C), which preceded vacuum drying. Such treatment can exhaust the RBC energy sources and produce metabolic waste that can further compromise cellular recovery during drying and storage. In contrast, previous experiments did not require prolonged incubation of RBC specimens, and vacuum drying took place shortly after sample preparation.

One limitation of this study is that it does not provide data regarding intracellular GSH levels of BSO-treated and untreated RBCs. Several attempts to determine GSH concentrations by colorimetric assays or flow cytometry failed to provide a reliable measurement of that metabolite. In the absence of GSH measurement, it is impossible to determine whether the differences in hemolysis levels between BSO-treated and untreated RBCs derive from glutathione depletion.

5.5 CONCLUSION

This chapter contributes to this dissertation by supporting previous findings presented in Chapters 3 and 4. It supports the observation that ROS formation in vacuum-dried RBCs is not necessarily the consequence of Hb oxidation. Data acquired from glucose-treated RBCs at two residual moisture contents strengthen the argument that different mechanisms of damage take place at different levels of dehydration.

This chapter also expands our understanding regarding the complexity of mechanisms capable of affecting cellular recovery following desiccation stress. In regard to glucose, factors like the residual moisture content are crucial for its role as a cellular

protectant. In the case of glutathione, vacuum-dried RBCs may resemble pathological RBCs in a sense of unique susceptibility to GSH-induced oxidative injury due to heme degradation. These factors need to be considered in future attempts to stabilize RBCs in the dried state.

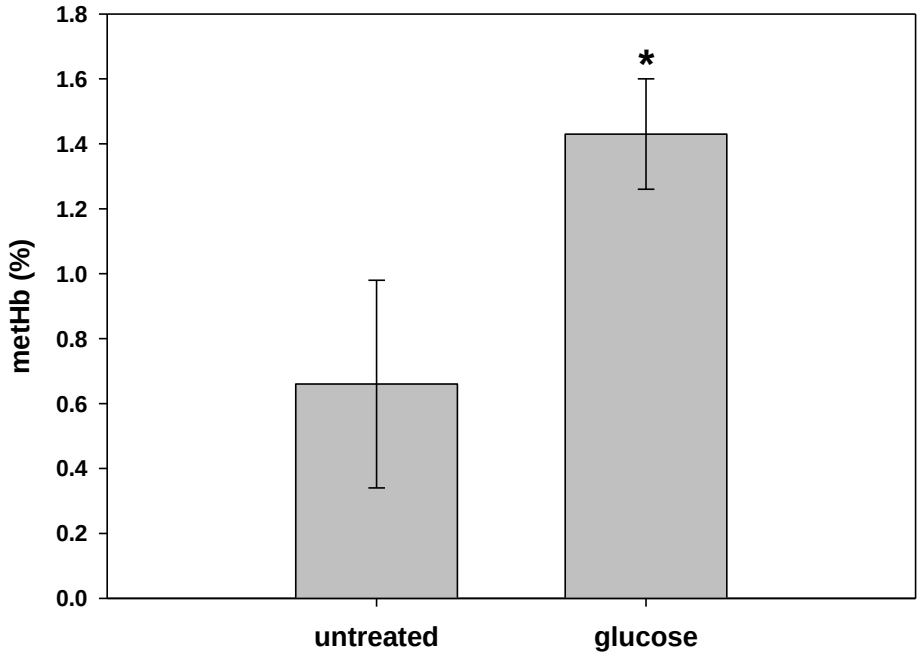

Figure 5.1. Vacuum-drying of glucose-treated RBCs: Hb oxidation in partially dehydrated samples (5.8 ± 0.3 g H_2O/g dry weight). Glucose-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Rate of Hb oxidation is represented as percent metHb. Error bars represent standard deviation (SD). Asterisk symbol (*) denotes a significant difference (p=0.002) between the two RBC groups (n=7).

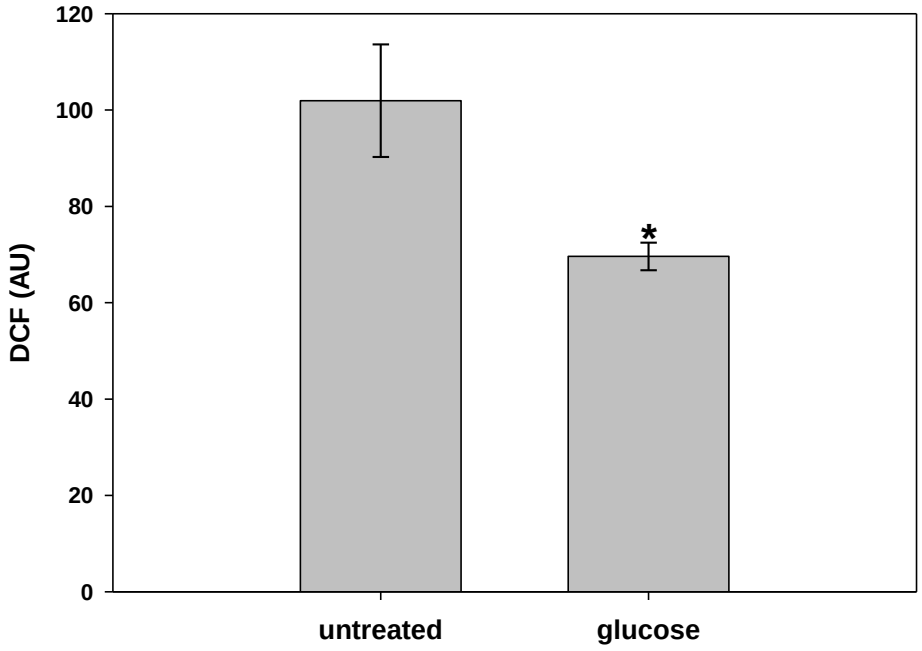

Figure 5.2. Vacuum-drying of glucose-treated RBCs: ROS formation in partially dehydrated samples (5.8 ± 0.3 g H_2O/g dry weight). Glucose-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). ROS formation is represented as DCF fluorescence (arbitrary units). Error bars represent standard deviation (SD). Asterisk symbol (*) denotes a significant difference (p=0.002) between the two RBC groups (n=7).

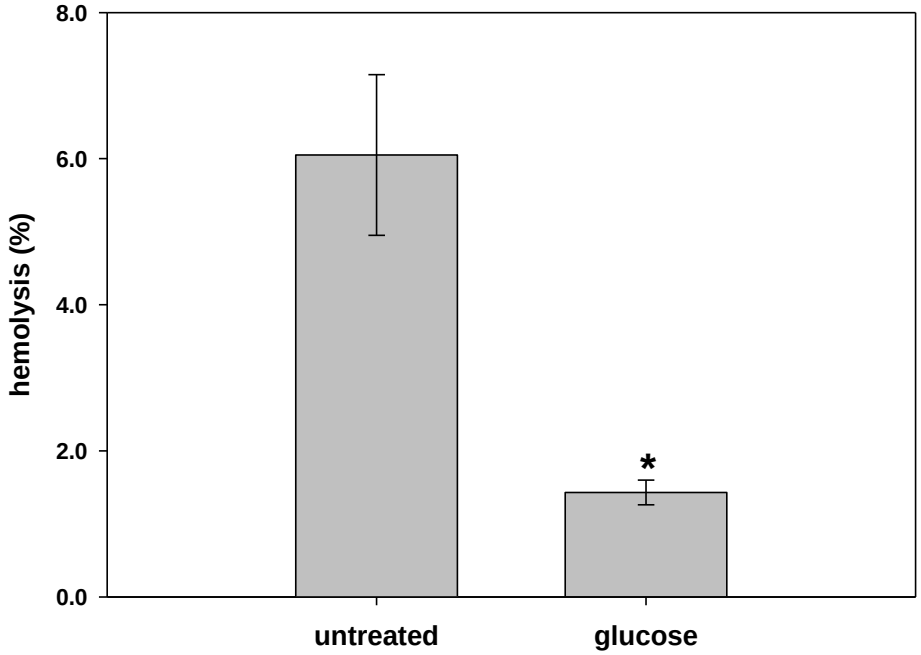

Figure 5.3. Vacuum-drying of glucose-treated RBCs: Hemolysis in partially dehydrated samples (5.8 ± 0.3 g H_2O/g dry weight). Glucose-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Cell integrity is represented as percent hemolysis. Error bars represent standard deviation (SD). Asterisk symbol (*) denotes a significant difference (p=0.002) between the two RBC groups (n=7).

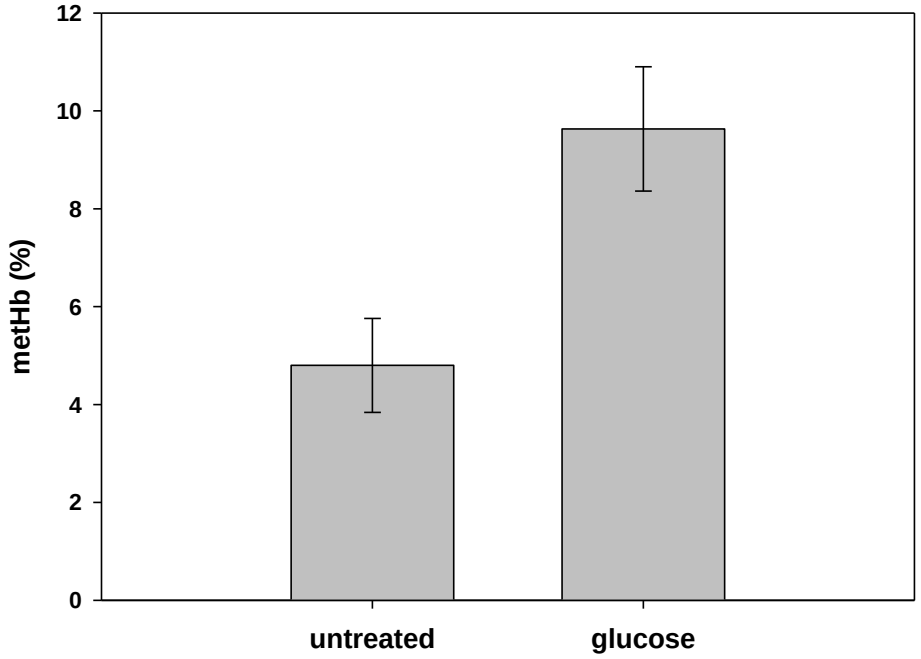

Figure 5.4. Vacuum-drying of glucose-treated RBCs: Hb oxidation in relatively dry samples (2.8 ± 0.5 g H_2O/g dry weight). Glucose-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Rate of Hb oxidation is represented as percent metHb. Error bars represent standard deviation (SD) (n=2).

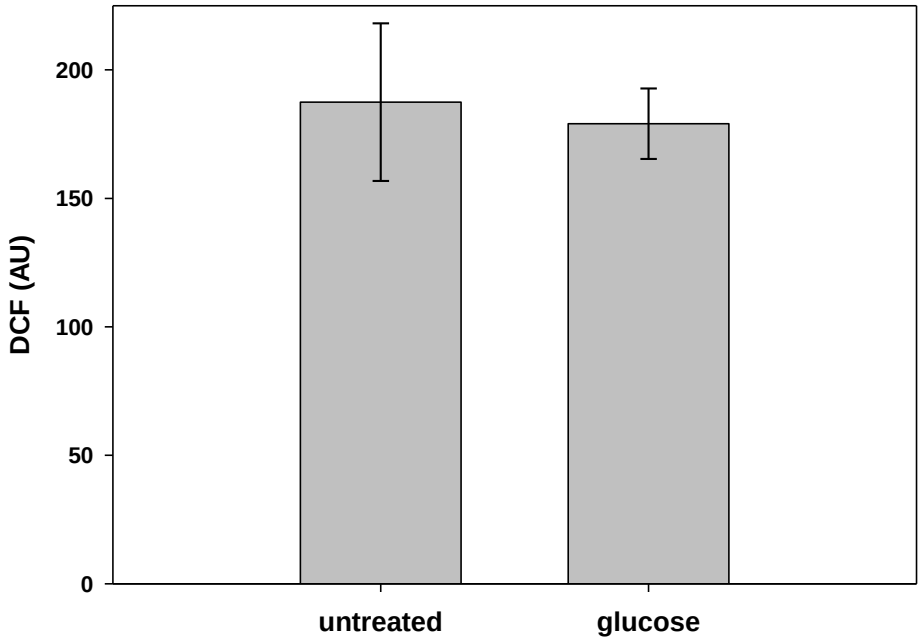

Figure 5.5. Vacuum-drying of glucose-treated RBCs: ROS formation in relatively dry samples (2.8 ± 0.5 g H_2O/g dry weight). Glucose-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). ROS formation is represented as DCF fluorescence (arbitrary units). Error bars represent standard deviation (SD) (n=2).

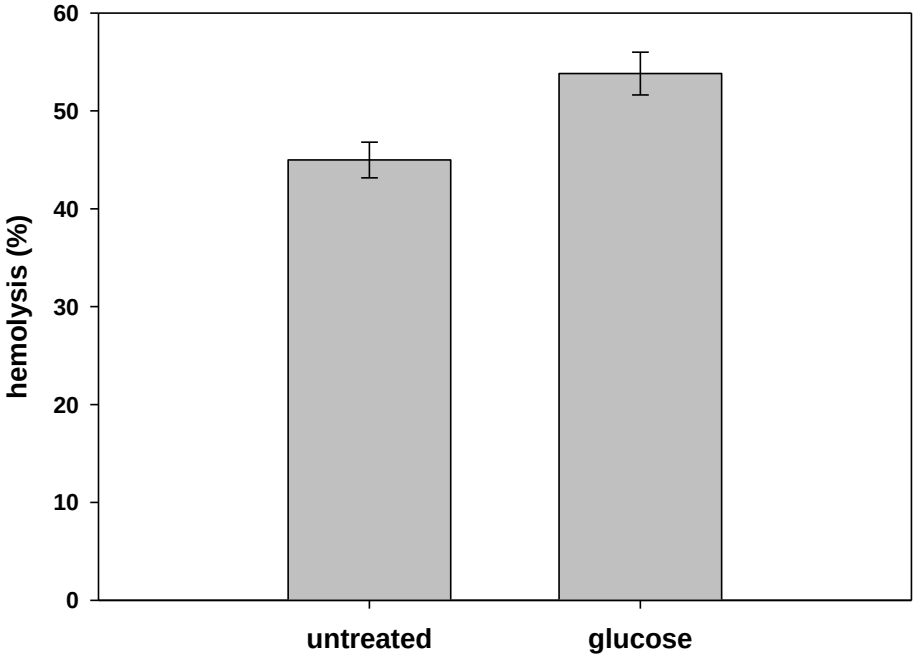

Figure 5.6. Vacuum-drying of glucose-treated RBCs: Hemolysis in relatively dry samples (2.8 ± 0.5 g H_2O/g dry weight). Glucose-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Cell integrity is represented as percent hemolysis. Error bars represent standard deviation (SD) (n=2).

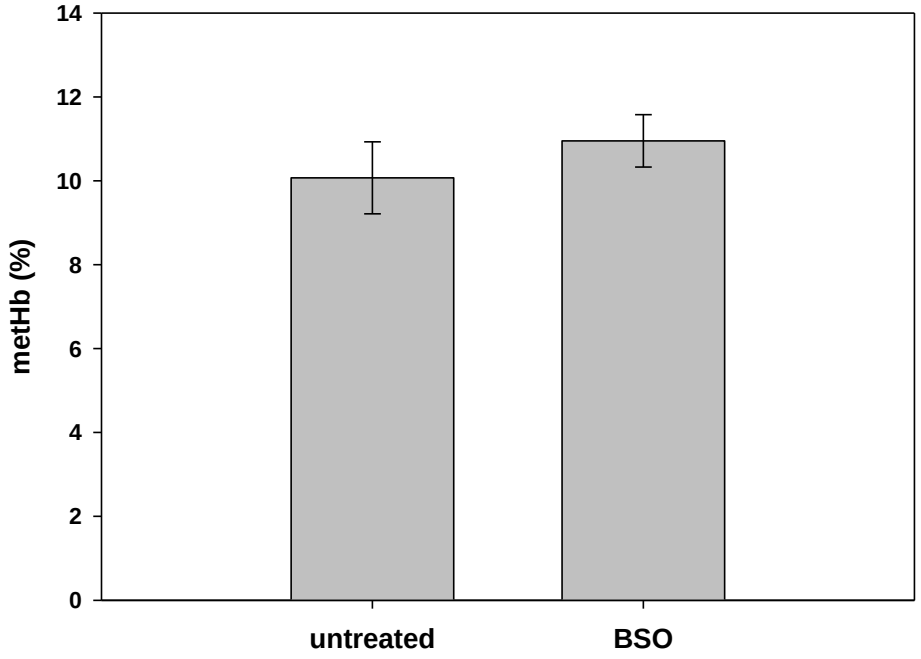

Figure 5.7. Vacuum-drying of BSO-treated RBCs: Hb oxidation in partially dehydrated samples (5.0 ± 0.5 g H_2O/g dry weight). BSO-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Rate of Hb oxidation is represented as percent metHb. Error bars represent standard deviation (SD) (n=8).

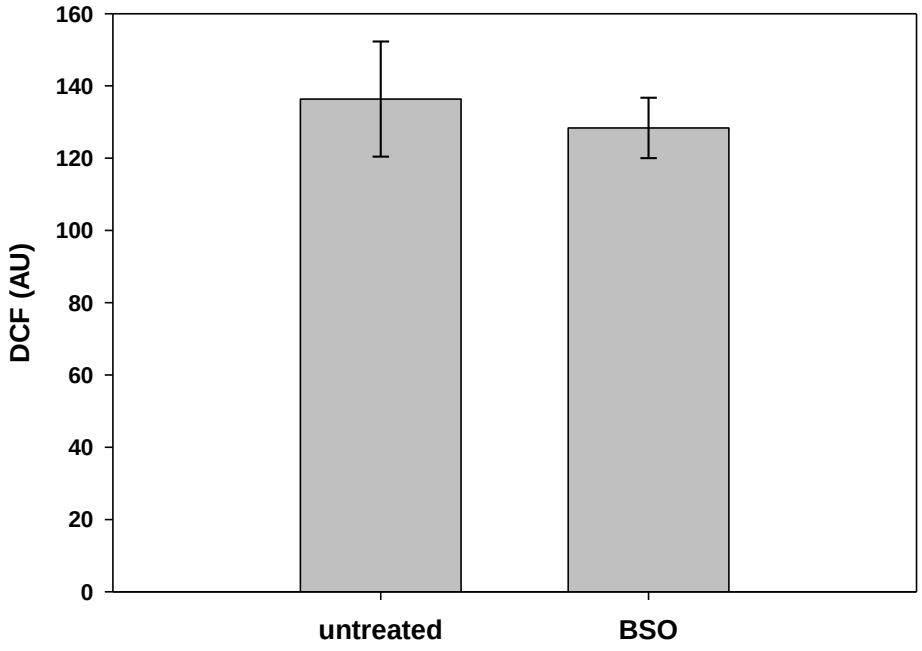

Figure 5.8. Vacuum-drying of BSO-treated RBCs: ROS formation in partially dehydrated samples (5.0 ± 0.5 g H_2O/g dry weight). BSO-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). ROS formation is represented as DCF fluorescence (arbitrary units). Error bars represent standard deviation (SD) (n=8).

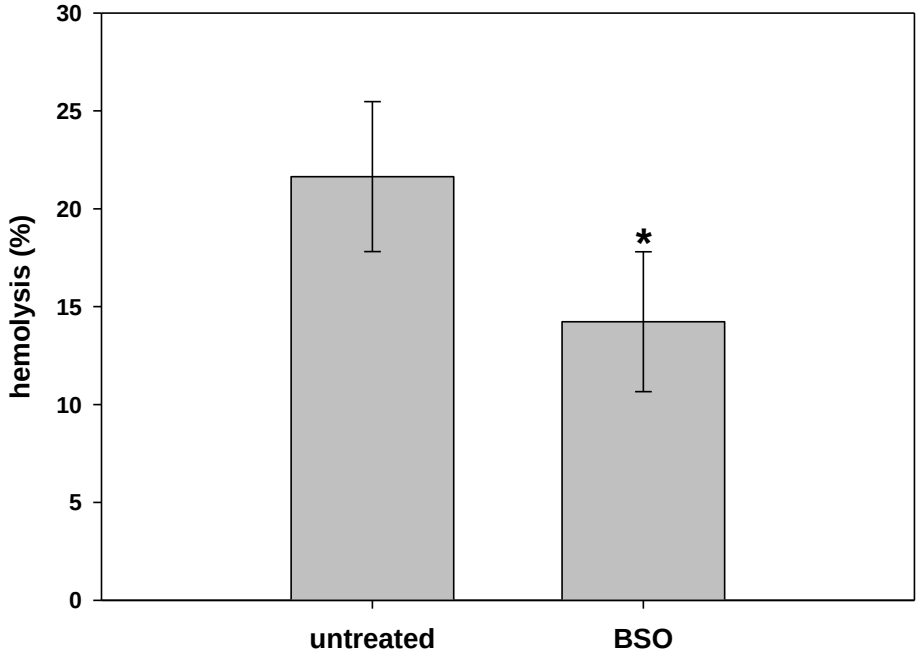

Figure 5.9. Vacuum-drying of BSO-treated RBCs: Hemolysis in partially dehydrated samples (5.0 ± 0.5 g H_2O/g dry weight). BSO-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Cell integrity is represented as percent hemolysis. Error bars represent standard deviation (SD). Asterisk symbol (*) denotes a significant difference (p=0.002) between the two RBC groups (n=8).

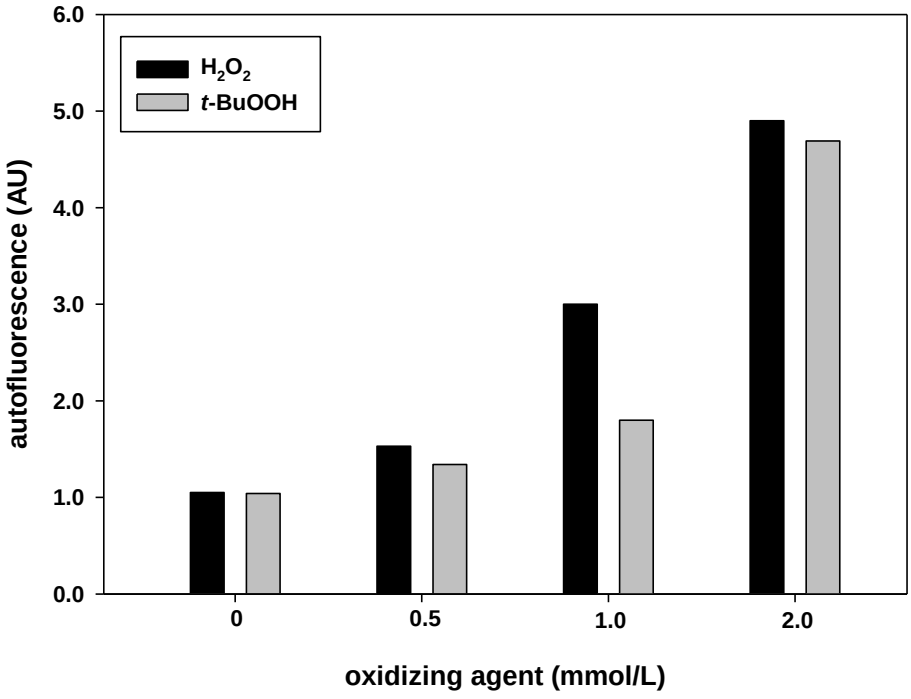

Figure 5.10. Heme degradation in response to oxidative challenge. RBC suspensions (15 % HCT) were incubated (15 min, 37 °C) in the presence of oxidizing agents, H_2O_2 or t-BuOOH, at selected concentrations of 0.5, 1 and 2 mmol/L. Heme degradation is demonstrated by flow cytometric analysis of RBC autofluorescence. Data derives from mean fluorescence intensity (arbitrary units) of non oxidized and oxidized RBC samples (n=1).

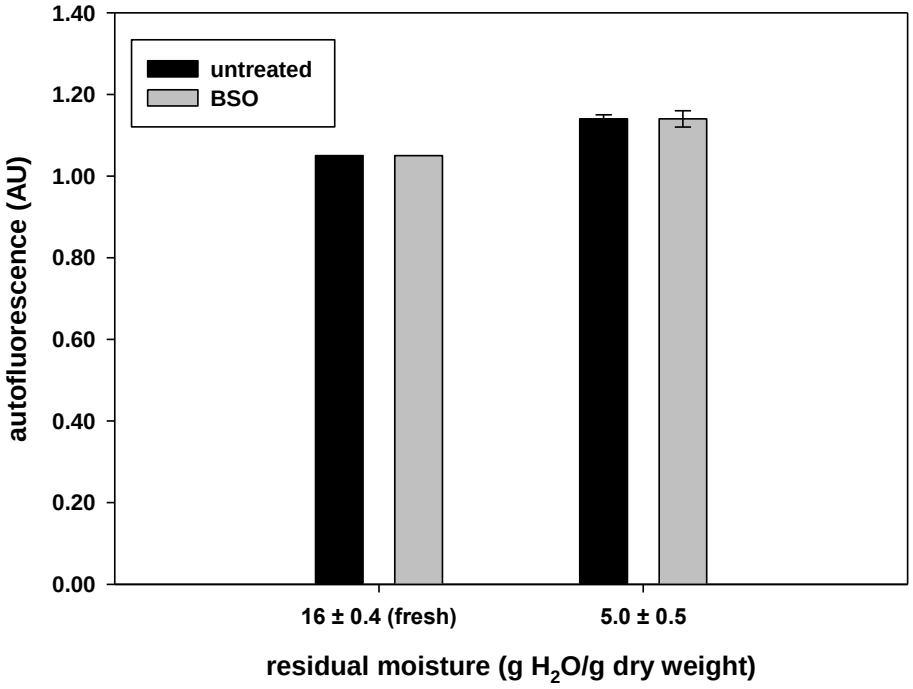

Figure 5.11. Heme degradation in vacuum-dried RBCs. BSO-treated RBCs (5 mmol/L in cell suspension) and untreated RBCs (controls) were vacuum-dried for 70-76 h and then stored at 25 °C for additional 96 h (total period of 7 days). Heme degradation is demonstrated by flow cytometric analysis of autofluorescence in fresh (16 ± 0.4 g H_2O/g dry weight) and vacuum-dried (5.0 ± 0.5 g H_2O/g dry weight) RBCs. Error bars represent standard deviation (SD). The mean fluorescence intensity of vacuum-dried RBCs (control and BSO-treated) was significantly ($p<0.001$) higher than that of fresh RBCs (n(fresh)=4; n(vacuum-dried)=8).

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CHAPTER 6: General discussion and conclusion

6.1 HYPOTHESIS

This dissertation examined the incidence of RBC oxidative injury during desiccation stress from the hemoglobin point of view. The original hypothesis stated that "desiccation stress in RBCs is accompanied with non-physiological oxidation of Hb and, consequently, the release of toxic products capable of compromising cell integrity through unrestricted oxidation of vital cellular components including the plasma membrane". Most elements of this hypothesis are supported by the results; however, some elements are incomplete.

The first element "desiccation stress in RBCs is accompanied with non-physiological oxidation of Hb" is strongly supported in this work and best demonstrated by Figure 3.1, which shows the correlation between water loss and metHb formation. This element is also supported by similar studies that demonstrated non-physiological levels of metHb in lyophilized RBCs [1, 2].

The second element refers to the release of toxic products (i.e. superoxide anions and metHb) by oxidized Hb. This element was supported by the increases in metHb and ROS levels seen in vacuum-dried RBCs (Figures 3.4, 3.5, 3.6, 4.3 and 4.6). However, two observations have challenged the hypothesis. The first relates to the toxicity of intracellular metHb, and the second ascribes to the origin of the ROS monitored in vacuum-dried RBCs.

Data presented in Chapter 4 clearly demonstrated that conversion of the RBC Hb to metHb prior to vacuum-drying render these cells more resistant to desiccation-induced injury in terms of lipid peroxidation (Figure 4.8) and hemolysis (Figure 4.7). In other

words, having non-physiological levels of intracellular metHb is not necessarily toxic. These results may appear to contradict data presented in Figure 3.3, which shows a strong correlation between metHb and hemolysis. However, there is a fundamental difference between the two cases. In metHb RBCs (Figure 4.7), Hb oxidation was performed in a controlled manner prior to vacuum-drying using sodium nitrite. ROS generated by this reaction (i.e. hemoglobin-nitrite) are eventually reduced to water [3]. In the other case (Figure 3.3), Hb oxidation and the subsequent release of superoxide anions took place during drying in an uncontrolled manner, which could have contributed to hemolysis.

ROS monitored in vacuum-dried RBCs were initially assumed to be the product of Hb oxidation. This assumption was based on previous studies, which regarded the autoxidation of hemoglobin as the prime source for intracellular ROS [4]. Data presented in chapters 4 (Figures 4.3 and 4.6) and 5 reveal that ROS formation in vacuum-dried RBCs is not directly correlated with Hb oxidation. On the contrary, an inverse correlation between Hb oxidation and ROS formation was observed with glucose-treated RBCs (Figures 5.1 and 5.2).

As discussed in chapter 4, the choice of DCFHA for monitoring ROS in vacuum-dried RBCs may not have been specific enough to distinguish Hb-derived free radicals (i.e. superoxide anions, hydrogen peroxide) from other cellular or extracellular sources including membrane-derived lipid hydroperoxides, peroxynitrite, and metabolic waste products. Therefore, "the release of toxic products by oxidized Hb", as stated in the hypothesis, requires further investigation because toxicity could have been derived by other factors capable of inducing Hb oxidative injury. One source is lipid peroxidation products, which were monitored in vacuum-dried RBCs (Figure 4.8). Lipid hydroperoxide toxicity was demonstrated several times in this work by the use of an

analog, t-BuOOH (Figures 3.4, 4.4, and 5.10), which was shown to induce Hb oxidation and heme degradation. While this study provides evidences for desiccation-induced Hb oxidation and membrane lipid peroxidation, the interactions between the two are not clear and yet to be determined.

Another aspect that is not addressed in the hypothesis is that Hb-induced cellular injury in vacuum-dried RBCs is strongly affected by the residual moisture content. Data presented in Chapter 4 clearly show that modifications in the Hb ligand state (i.e. metHb RBCs) strongly affect cell integrity in partially dehydrated samples, whereas minor effects were observed with relatively dried samples (Figure 4.7). Correspondently, membrane lipid peroxidation was more pronounced in partially dehydrated RBC samples than in relatively dry samples (Figure 4.8). These observations are not limited to oxidative injury, because similar pattern was observed with other pharmacological treatments including glucose (Figures 5.3 and 5.6) and BSO (Figure 5.9).

In conclusion, this work suggests that desiccation stress in RBCs is accompanied with oxidative injury characterized by non-physiological oxidation of the Hb, formation of intercellular ROS and membrane lipid peroxidation. Hb-induced oxidative injury is water dependent corresponding to earlier stages of water loss. As water levels continue to drop, the cell suffers increases in intracellular solute concentrations that could potentially increase the rate of redox reactions. However, the concurrent increase in intracellular viscosity is likely to reduce molecular motion and consequently compromise chemical reactions. At this stage of dehydration, cellular injury is probably the result of physical and mechanical stress. Figure 6.1 summarizes this model.

6.2 RELEVANCE TO THE FIELDS OF CELL PRESERVATION AND FUTURE STUDY

Stabilization of RBCs in the dried state is not a new concept, as studies can be dated back to the early works of Harold T. Meryman [5, 6] over five decades ago. Meryman's work was succeeded by various attempts to enhance desiccation tolerance in RBCs using various approaches (for review see Kanias and Acker [7]) including freeze-drying of trehalose-loaded RBCs [1, 2, 8]. Despite the extensive efforts, little success has been documented in that field. At present, no reconstituted RBCs meet the standard for transfusion.

Studies of desiccation-induced cellular injury in mammalian cells have focused primarily on membrane damage in response to physical and mechanical stresses [2, 9-11]. Little attention has been given to other cellular organelles that are susceptible to desiccation stress or to other factors capable of compromising cellular recovery. While oxidative injury is a major threat to survival of many anhydrobiots [12, 13], this issue has been poorly recognized in the field of mammalian cell preservation.

This is the first dissertation that is completely dedicated to studying the correlation between desiccation and oxidative injury in mammalian cells, and as such it introduces the field of free radical biology to the disciplines of cell preservation and biobanking. Previous studies of desiccated RBCs lack understanding of biochemical pathways capable of inducing oxidative injury and, consequently, compromise cellular recovery. This study clarifies these issues and indicates that oxidation, as a stress factor, must be considered in future studies.

Further clarification of the RBC oxidative injury during desiccation can take several directions. As discussed in Chapter 4, ferrylHb is a strong oxidizing agent capable of mediating cellular injury in RBCs. Monitoring for ferrylHb in reconstituted RBCs can elucidate the nature of Hb redox reactions that take place during desiccation. Another direction can further examine changes in the RBC antioxidant activity (catalase, glutathione and its related enzymes) during desiccation. Other studies can focus on methods to counteract oxidative injury by screening for potential antioxidants, or by developing methods for drying and storage under anaerobic conditions.

Data acquired for this dissertation went beyond testing the hypothesis to expand our understanding regarding *ex vivo* preservation of RBCs and oxidative injury. First, dedicating a review article about Hb oxidation and its implications on biopreservation of RBCs [14]. Second, characterizing the oxidative injury during trehalose loading into RBCs [15]. Third, addressing critical errors in the measurement of lipid peroxidation of trehalose-loaded RBCs published by another research group [16]. Fourth, acknowledging other stress factors (glucose and glutathione depletion) capable of affecting cellular recovery in vacuum-dried RBCs (Chapter 5).

This study also introduced various assays for monitoring oxidative stress in banked and reconstituted RBCs. The majority of these assays were successfully adopted from the field of RBC disorders, such as anemia and methemoglobinemia. These assays allow for monitoring changes in the RBC oxidative state including Hb autoxidation, ROS formation and membrane lipid peroxidation. Future use of such assays would contribute to basic research in the field of RBC storage injuries, or as an additional tool for the current practice of RBC quality monitoring in transfusion medicine.

Finally, this study has shown that oxidative injury in vacuum-dried RBCs is water dependent. This finding is of great importance, as it can significantly affect future design of RBC products. Preserving RBCs at a medium range of residual moisture content can reduce cellular injury in response to osmotic pressure, but at the same time requires pharmacological treatment to overcome oxidative injury and sustain proper metabolic activity. RBC storage under lower residual moisture content can significantly reduce cellular injury in response to metabolic activity, but at the same time requires the introduction of glass-forming cell protectants to protect against physical and mechanical stress. Ultimately, this should be the state of the art of RBC preservation because the premise of anhydrobiosis relies on suspending, rather than sustaining, animation. Nevertheless, characterizing the correlation between desiccation and oxidative injury is a major milestone in achieving this goal.

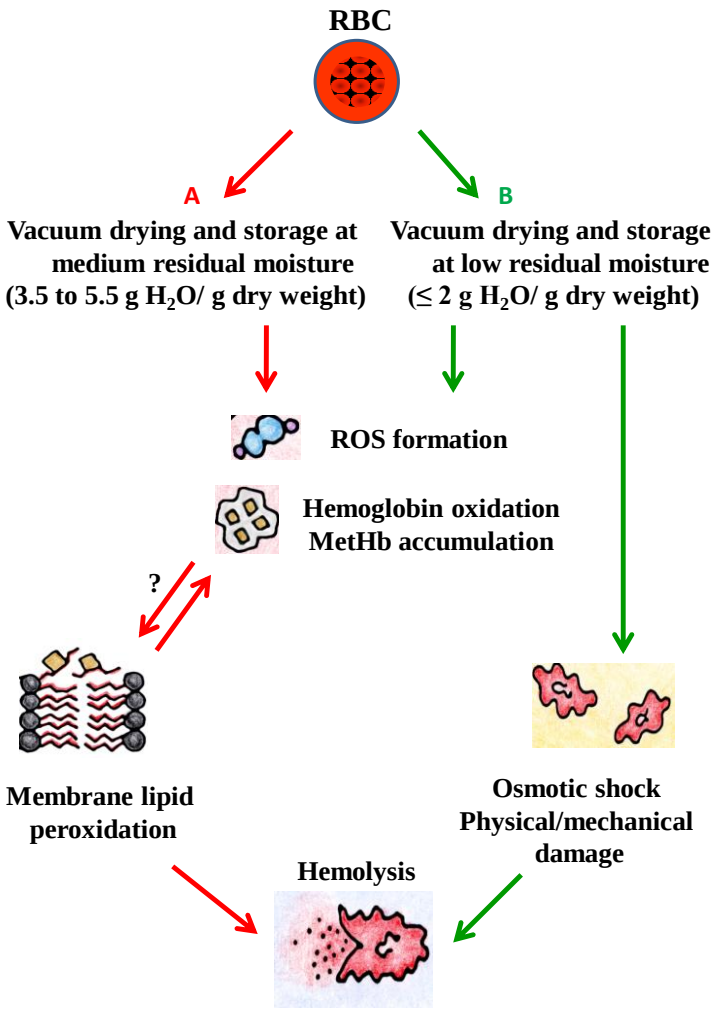

Figure 6.1. Proposed model for hemoglobin-induced cellular injury in vacuum-dried RBCs. **A**. Hemoglobin-induced oxidative injury is water dependent corresponding to earlier stages of dehydration (3.5 to 5.5 g H_2O/ g dry weight). This range of residual moisture content is characterized with a drastic increase in the rate of hemoglobin oxidation, ROS formation, membrane lipid peroxidation, and, consequently, hemolysis. The exact mechanism of damage is not clear, and can be related to redox reactions between oxidized membrane lipids and hemoglobins. Pharmacological treatments of the hemoglobin oxygen binding site at this range of residual moisture content can significantly affect the rates of membrane lipid peroxidation and hemolysis. **B**. Increased cellular viscosity compromises biochemical reactions at lower residual moisture contents (≤ 2 g H_2O/ g dry weight) and cellular injury is likely the result of physical and mechanical stress. Pharmacological treatments of the hemoglobin oxygen binding site at this range of residual moisture content do not affect the rates of membrane lipid peroxidation and hemolysis. Moreover, these RBCs contain low levels of oxidized lipids suggesting that lipid peroxidation does not play a major role in mediating membrane damage and hemolysis at this range of residual moisture content.

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