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A NOVEL RED BLOOD CELL SUBSTITUTE BASED ON CROSSLINKED HEMOGLOBIN, SUPEROXIDE DISMUTASE, AND CATALASE

by

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Abstract

Modified hemoglobin red blood cell substitutes have a number of potential areas of application. In some of these applications, it will be important to lessen the pro-oxidant effects of hemoglobin and potential free radical-mediated toxicity. This research introduces a novel modified hemoglobin that is based on intermolecularly crosslinking hemoglobin, superoxide dismutase and catalase (PolyHb-SOD-CAT) with the bifunctional agent, glutaraldehyde. Superoxide dismutase and catalase catalyze the breakdown of superoxide radical and hydrogen peroxide respectively. Studies of structural and functional parameters reveal that PolyHb-SOD-CAT retains superoxide dismutase and catalase enzymatic activity, and consists of a mixture of molecular species ranging in molecular size and protein composition. Circulation time studies of PolyHb-SOD-CAT in rats show that hemoglobin, superoxide dismutase and catalase possess longer circulatory half-lives as compared to the free forms of these proteins. Studies also show that PolyHb-SOD-CAT prevents the formation of methemoglobin, ferrylhemoglobin, hydroxyl radical, free iron, and lipid peroxidation. Ischemia-reperfusion studies using isolated perfused hindlimbs and intestine of rat show that PolyHb-SOD-CAT reduced the formation of hydroxyl radical compared to PolyHb. Altogether, these results suggest that PolyHb-SOD-CAT is a potentially safer modified hemoglobin oxygen carrier by virtue of its ability to detoxify reactive oxygen species, and reduced propensity to promote and participate in oxidative processes.

Resume

Les substituts d'érythrocytes à base d'hémoglobine modifiée se prêtent à un certain nombre d'applications. Pour certaines, il faudra toutefois réduire les effets pro-oxydants de l'hémoglobine et le potentiel de toxicité à médiation radicalaire. Cette recherche porte sur un type d'hémoglobine modifiée qui combine l'hémoglobine et les enzymes antioxydants superoxyde-dismutase et catalase. Elle porte plus précisément sur une méthode qui fait intervenir les liaisons croisées intermoléculaires de l'hémoglobine, de la superoxyde-dismutase et de la catalase (PolyHb-SOD-CAT) avec l'agent bifonctionnel glutaraldéhyde. La superoxyde-dismutase et la catalase catalysent respectivement le fractionnement du radical superoxyde et du peroxyde d'hydrogène. Ces espèces d'oxygène réactive entrent en jeu dans les mécanismes radicalaires provoqués par les hémoglobines modifiées et dans d'autres processus de stress oxydatif *in vivo*. Les études des paramètres structuraux et fonctionnels révèlent que la PolyHb-SOD-CAT conserve l'activité enzymatique de la superoxyde-dismutase et de la catalase et est un mélange d'espèces moléculaires de taille moléculaire et de composition protéinique variables. Les études du temps de circulation de la PolyHb-SOD-CAT chez le rat démontrent que l'hémoglobine, la superoxyde-dismutase et la catalase ont des demie-vies circulatoires plus longues que les formes libres de ces protéines. Elles révèlent également que la PolyHb-SOD-CAT empêche la formation de la méthémoglobine, de la ferrylhémoglobine, du radical hydroxyle et du fer libre, et qu'elle prévient ainsi la peroxydation des lipides. Les études de reperfusion au cours d'une ischémie portant sur les membres antérieurs et l'intestin isolés et perfusés de

rats démontrent que la PolyHb-SOD-CAT réduit davantage la formation de radicaux hydroxyles que la PolyHb. En conclusion, ces résultats portent à croire que la PolyHb-SOD-CAT pourrait être plus sûre comme porteur d'oxygène à base d'hémoglobine modifiée, du fait de ses propriétés détoxifiantes à l'égard des espèces d'oxygène réactive et de sa moindre propension à promouvoir activement les processus oxydatifs.

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Table of Contents

Chapter 3

Structural and Functional Aspects of a Novel Red Blood Cell Substitute based on Crosslinked Hemoglobin- Superoxide Dismutase- Catalase

Chapter 4

Absence of Hemoprotein-Associated Free Radical Events Following Oxidant Challenge of Crosslinked Hemoglobin- Superoxide Dismutase-Catalase

Chapter 5

Reduction of Hydroxyl Radical Generation in a Rat Hindlimb Model of Ischemia-Reperfusion Using Crosslinked Hemoglobin-Superoxide Dismutase-Catalase

Chapter 6

Crosslinked Hemoglobin-Superoxide Dismutase-Catalase Scavenges Free Radicals in a Rat Model of Intestinal Ischemia-Reperfusion Injury

Chapter 7

General Discussion and Conclusions

List of Figures

List of abbreviations

m	milli (x 10^{-3})
μ	micro (x 10^{-6})
n	nano (x 10^{-9})
p	pico (x 10^{-15})
k	kilo (x 10^{3})
mU/ml	milliunits per milliliter
g/dl	grams per deciliter
units/ml	units per milliliter
μg/l	micrograms per liter
μM	micromolar
mM	millimolar
nm	nanometer
nM	nanomolar
M	molar
pmol	picomole
min	minute
hr	hour
°C	degrees Celsius
%	percent
SD	standard deviation
SE	standard error
Hb	hemoglobin
SOD	superoxide dismutase

CAT	catalase
PolyHb-SOD-CAT	polymerized hemoglobin, SOD, and catalase
PolyHb	polymerized hemoglobin
FMDA	fumaryl-monodibromoaspirin
DBBF	bis (3,5-dibromosalicyl) fumarate
NFPLP	nor-2-formylpyridoxl-5'-phosphate
HPLC	high performance liquid chromatography
kD	kilo Daltons
4-HB	4-hydroxybenzoate
DHBA	dihydroxybenzoate
EDTA	ethylenediaminetetraacetic acid
$O_2^{\bullet-}$	superoxide anion radical or superoxide
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite anion
NO	nitric oxide
PUFA	polyunsaturated fatty acid

Preface

The thesis is composed of seven chapters. Chapter 1 is a general literature review that provides background information pertaining to the thesis. Chapter 7 includes general conclusions and claims to original research. For chapters 2, 3, 4, 5, and 6, I have taken advantage of the option provided by section B.2 of the "Guidelines Concerning Thesis Preparation" which states:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no**

circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

Chapter 2 has been published in *Biomaterials, Artificial Cells, and Immobilization Biotechnology 21: 671-675, 1993.* Chapter 5 has been published in *Artificial Cells, Blood Substitutes and Immobilization Biotechnology 25:(in press), 1997.*

Chapter 6 has been published in *Artificial Cells, Blood Substitutes and Immobilization Biotechnology 25: (in press), 1997.* I appear as second author in this latter publication. My responsibilities consisted of: performing literature search, providing perfusate preparation and sample analysis methodologies, performing data analysis and data presentation. Dr. Saleem Razack, Division of Pediatric Critical Care Medicine, Montreal Children's Hospital, the first author of the paper, performed surgical manipulations and sample collection. Dr. Chang discussed the planning of the research and the interpretation of the results. The manuscript was prepared as a combined effort of Dr. S. Razack, Dr. T.M.S. Chang, and myself. Chapters 3 and 4 have been prepared for submission.

CHAPTER 1

Introduction

1.1 Red Blood Cell Substitutes

1.1.1 Need For a Red Blood Cell Substitute

Brief historical review of transfusion medicine

Red blood cell transfusion therapy is one of the most significant developments in medical history. In 1616, William Harvey's discovery of the circulation of blood stimulated the modern era of blood replacement research [1,2]. In 1665, physician Richard Lower is believed to have recorded the first successful animal-to-animal blood transfusion by keeping dogs alive with blood from other dogs [1]. Soon after, in 1667, both Richard Lower in England and Jean-Baptiste Denis in France separately reported on blood transfusions from lambs to humans [1,3]. In 1678, animal blood transfusions in humans became illegal in both countries as dangerous consequences were recognized. About 150 years later, the practice of transfusion was revived by an English physiologist and obstetrician named James Blundell. In 1818, he performed the first successful transfusions of human blood to patients suffering from postpartum hemorrhage [1-3]. Based on a series of animal experiments, Blundell stated that only human blood should be used in humans. Unfortunately, the use of animal blood continued during the late 1800's with disastrous results. During this period, adverse transfusion reactions were also observed with human blood. In 1900, Karl Landsteiner and his colleagues discovered the A, B, and O human blood groups [1-3]. This monumental finding was followed by the discovery of the AB blood group in 1902 by DeCastello and Struli. In 1907, Reuben Ottenberg, reported the first blood transfusion

using blood typing and crossmatching [1-3]. Another important landmark in blood transfusion occurred in 1914 when Hustin, along with others, reported the use of sodium citrate and glucose as a diluent and anticoagulant solution for transfusions. During the 1900's, significant advances emerged in areas related to blood collection, storage, fractionation, disease screening tests, and blood banking procedures. [1-3]

Potential problems associated with the use of red blood cells

The practice of red cell transfusion is safer than ever. Red blood cells are used to treat accident victims, patients undergoing surgery, and patients receiving treatment for leukemia, cancer, or other diseases such as sickle cell anemia. Though widely used, the risks and benefits of transfusion therapy must be carefully evaluated for each patient [4].

Several drawbacks or concerns associated with the use of red blood cells [1-3] include (i) the absolute requirement for typing and crossmatching of blood groups. Compatibility testing of ABO blood groups still does not eliminate the risk for transfusion reactions due to other unidentified mismatches or potential antibody formation against red cell antigens in recipients who receive repeated blood transfusions (ii) storage limitations of red blood cells. Red blood cells can be stored under refrigeration for 35-42 days with current anticoagulant preservatives. Alternatively, red blood cells can be frozen for a period of 10 years. However, after thawing, frozen red cells must be used within 24 hours. (iii) availability of donor blood.

The demand for red blood cells exceeds its supply due to an increase in the use of transfusions related to recognition of increased safety, increase in special surgical procedures that require high blood transfusions, and the perceived fear of contracting disease by donating blood contributes to decreased number of donations. Availability of donor blood may especially represent an important obstacle in situations such as war or disasters. (iv) the potential transmission of disease-causing microorganisms. The risk of disease contraction remains the greatest source of anxiety in the population. Each unit of blood is subject to screening test for different types of viruses such as; hepatitis (B, C, non-A non-B), Human Immunodeficiency Virus (HIV), Human T Lymphotropic Virus (HTLV-I, -II), syphilis, and others. However, the discovery of new transfusion-related disease is likely to continue.

These shortcomings have traditionally stimulated to varying degrees research interest in red blood cell substitutes [2,5]. In the mid 1980's, the most important stimulus in blood substitute research emerged. It was recognized that HIV, the virus causing AIDS, could be transmitted by blood transfusions [6]. This finding substantially intensified academic and industrial research effort in the field of red blood cell substitutes. Today, careful blood collection and testing procedures have substantially reduced the risk of HIV transmission. Presently, red blood cells remain the best material for transfusion in situations requiring restoration or maintenance of oxygen-carrying capacity. Many advantages may be gained with the development of red blood cell substitutes, however, in order to gain public and clinical acceptance they must prove to

be effective and safe.

1.1.2 Overview of Red Blood Cell Substitutes

General classification

Red blood cell substitutes are advantageous since they do not have blood group antigens and viruses, and have longer storage possibilities. The most important functions of a red blood cell substitute are oxygen transport and plasma volume expansion. Several red cell substitutes have oxygenation characteristics similar to normal red cells and possess useful circulation times. Furthermore, the oncotic pressure and viscosity is similar to plasma. Several promising substitutes with these characteristics are currently available [2,6,7].

The two main approaches of red blood cell substitute technology are perfluorochemicals and hemoglobin-based substitutes. Firstly, perflurochemicals (PFCs) are hydrocarbons in which the hydrogen atoms have been substituted with fluorines. PFCs are chemically inert compounds that can dissolve oxygen, carbon dioxide, and other gases. The amount of gas dissolved is directly proportional to the partial pressure of the gas. In 1966, Clark demonstrated that PFCs could dissolve enough oxygen to allow the survival of liquid-breathing mice [8]. PFCs are insoluble in water, therefore they must be emulsified prior to intravenous use. Sloviter was first to develop a water-soluble emulsion using albumin [9], and Geyer first reported the survival of rats whose blood volume was completely replaced with PFCs [10]. In 1989,

the United States Food and Drug Administration (FDA) approved the use of a PFC preparation, Fluosol-DA, for percutaneous transcoronary angioplasty. In 1986, however, it was also shown that Fluosol-DA was an ineffective oxygen carrier in surgical patients with acute severe anemia [11]. A number of other promising PFCs are currently being developed. Further description of PFCs is beyond the scope of this thesis. Reviews on this subject are available [12,13].

Hemoglobin-based red cell substitutes are considered to be more "natural" replacements for red blood cells. Hemoglobin is the protein in red blood cells responsible for binding oxygen. Hemoglobin can not be used as a red blood cell substitute for reasons that will be discussed in section 1.2.2. In 1957, Chang began working on modifying hemoglobin with his studies on artificial red blood cells [14,15]. His early studies also included crosslinked and conjugated hemoglobin [15-17]. Today, there are two main categories of hemoglobin-based red blood cell substitutes, microencapsulated hemoglobin and crosslinked hemoglobin. The latter approach can be further subdivided into intramolecular, intermolecular, and conjugated hemoglobin. These approaches will be further described in section 1.2.3. Exchange transfusion and hemorrhagic shock resuscitation animal studies have demonstrated the oxygen delivery and volume expansion efficacy of modified hemoglobin, and clinical trials are ongoing in several centers [2,7,18,19]. The FDA's "Points to consider" publication emphasized the need for basic understanding of hemoglobin toxicity [20]. Thus, considerable research efforts currently focus on important safety concerns associated with the use of

modified hemoglobin oxygen carriers.

Potential clinical implications

There are several proposed clinical indications for a red cell substitute [2,6,8]. Some potential applications include resuscitation of hypovolemic shock, preoperative hemodilution for elective surgical procedures, treatment of ischemic disease, use in coronary angioplasty, organ preservation for transplantation, and others. It is unlikely that one type of red blood cell substitute will be able to fulfill all these potential indications. A more likely scenario for the future may be the use of different substitutes each capable of performing roles designed for specific clinical requirement.

1.2 Hemoglobin-based Red Blood Cell Substitutes

1.2.1 Hemoglobin: Structure and Function

Hemoglobin (~64,500 kD) is the oxygen-binding protein found in red blood cells. Human hemoglobin A (Hb A) is composed of two identical alpha chains (α) and two identical beta chains (β). The primary sequence of the human α chain (mw. ~15,126) consists of 141 amino acids. The β chain (mw. ~15,867) is composed of 146 residues [21]. The secondary structure describes the spatial arrangement of each globin chain due to interactions between residues located in close proximity along the same polypeptide. About 75 % of the amino acids of each chain are arranged in an alpha helical pattern. The β chain contains 8 helices which are designated A to H. The α

chain contains 7 helices which are also designated A to H, except it lacks the residues corresponding to the D helix. The length of these helices range from seven to twenty amino acids. The tertiary structure describes how each globin chain is folded into compact spherical or globular conformations in three dimensional space. The quaternary structure describes how the four globin chains of hemoglobin are arranged together. The globin chains of hemoglobin form a tetrahedral arrangement with a twofold axis of symmetry. This arrangement forms a central open cavity in the molecule which is lined with positively charged residues. There is minimal direct contact between the two α chains or the two β chains. Two interfaces between unlike subunits are assigned α_1-β_1 (and corresponding α_2-β_2) and α_2-β_1 (and corresponding α_1-β_2). The α_1-β_1 and α_2-β_2 interfaces remain virtually fixed during oxygenation, whereas there is considerable movement about the α_1-β_2, and α_2-β_2 interfaces. Based on the trigger hypothesis, binding of oxygen to iron induces a change in the tertiary structure of the globin chain. The changes in quaternary structure occur after two or three oxygen molecules have bound. The two quaternary conformations are assigned the deoxy (or tense) state and the oxy (or relaxed) state [21].

Each globin chain bears a iron-containing heme prosthetic group. Heme is a porphyrin ring structure (protoporphyrin IX), comprised of four pyrrole rings arranged in circular, planar array. Iron is bound to four nitrogen atoms of the ring and to the imidazole nitrogen of the 'proximal" histidine at position 87 for the α chain, and

position 92 for the β chain. Functional heme contains iron in the ferrous (Fe^{2+}) state. In this state, iron can share an electron to allow the binding of oxygen. When oxyhemoglobin releases oxygen, the iron remains in ferrous conformation. Methemoglobin contains ferric iron (Fe^{3+}), and thus does not possess the electron needed to bind oxygen. Heme is positioned between the E and F helices in a region surrounded by non-polar residues. This hydrophobic environment excludes water molecules, and thus protects ferrous iron from oxidation. The tetrameric conformation appears to assist in this function as well since αβ dimers and isolated globin chains autoxidize at a higher rate.

The primary function of hemoglobin is the transport of oxygen to tissues. At high partial pressures of oxygen (PO_2), hemoglobin loads oxygen while at low PO_2 oxygen is released. The oxygen dissociation curve of hemoglobin describes the uptake of oxygen by hemoglobin versus the partial pressure of oxygen. The P_{50} value represents the partial pressure of oxygen at which half the oxygen binding sites of hemoglobin are bound to oxygen. The sigmodial shape of the curve is due to the presence of binding cooperativity. This relates to the fact that the oxygenation of one subunit increases the oxygen affinity of other subunits. Inversely, the release of the first oxygen molecule from fully saturated hemoglobin facilities the release of subsequent molecules. The shape of the saturation curve permits nearly complete saturation of hemoglobin at PO_2

values present in the lung, and allows significant fraction of oxygen to be released at PO_2 values found normally in tissues.

Several important parameters that markedly affect the oxygen affinity of hemoglobin are pH, carbon dioxide (PCO_2), temperature, and 2,3 diphosphoglycerate (2,3 DPG). These factors affect the position of the oxygen saturation curve. Specifically, increases in temperature, hydrogen ion concentration, carbon dioxide, and 2,3 DPG all favour the deoxyhemoglobin conformation, thus produce a rightward shift in the saturation curve or a higher P_{50} value. The red cell metabolite, 2,3 DPG, fits between the two β-chains of deoxyhemoglobin. The two phosphate groups of this compound interact with the terminal amino groups of the β-chains while the carboxyl group interacts with lysine 82β, this tends to stabilize the deoxyhemoglobin conformation. When hemoglobin loads oxygen, established salt bridges in quaternary structure are broken and the beta chains are pulled together, expelling 2,3 DPG. The changes in oxygen affinity due to pH changes is known as the Bohr effect. A decrease in pH produces a decrease in oxygen affinity. This is related to the fact that deoxyhemoglobin binds hydrogen ions more readily that oxyhemoglobin. Carbon dioxide also affects oxygen affinity by a mechanism separate from its pH effects. Carbon dioxide binds to the N-terminal amino groups of the beta chains forming carbamino-hemoglobin. An increase in carbamino formation (or increase in CO_2) reduces oxygen affinity, whereas the loading of oxygen causes decrease in CO_2 binding.

Carbon dioxide is transported back to the lungs principally as bicarbonate ions with smaller fractions being transported as carbamino protein forms and as dissolved CO_2 in plasma.

1.2.2 Reasons Why Hemoglobin Requires Modification

In 1885, Benzcur was probably the first to inject hemoglobin subcutaneously into humans [2]. Later, in 1898, Von Starch reported his work on the subcutaneous injections of horse hemoglobin into humans [2]. No major side effects were observed. A number of hemoglobin infusions into animals during early 1900's preceded human experiments. In 1916, Sellards and Minot reported the first hemoglobin infusions in humans [2]. Most of their subjects experienced no negative side-effects, however a few experienced fever, chills, and nausea. After these studies, the intravenous administration of hemoglobin solutions in humans continued, but it rapidly became apparent that hemoglobin solutions were resulting in renal damage and blood coagulation. In 1967, Rabiner showed that contaminating red blood cell membranes ("red cell stroma") in hemoglobin solutions was the cause of nephrotoxicity and coagulation problems [22]. Rabiner's work was an important turning point since it was now believed that the previously noted toxicity of hemoglobin infusion was due mainly to the use of impure hemoglobin solutions. In 1978, Savitsky carried out a series of experiments on normal patients with purified (stroma-free) hemoglobin [23]. Savitsky observed a mild increase in blood pressure, bradycardia, and decreases in urine output

and creatinine clearance. Savitsky concluded that hemoglobin itself must be responsible for these effects since purified hemoglobin solutions were used.

It has been known for many years that animals survive complete exchange transfusion with hemoglobin solution, but cannot survive if given plasma substitute alone [24]. Hemoglobin solutions that contain red blood cell membrane contaminants are toxic in humans, and thus extensive purification of hemoglobin is required. However, there are other important limitations associated with the use of stroma-free hemoglobin as a red cell substitute: (1) Hemoglobin maintains its tetrameric form inside the red blood cell. However, outside the red blood cell, hemoglobin is rapidly broken down to dimers. These dimers are rapidly removed from the circulation by plasma haptoglobin or renal filtration; (2) Inside human red blood cells, hemoglobin interacts with 2,3 diphosphoglycerate (2,3 DPG). This interaction allows hemoglobin to have a low oxygen affinity. Outside the red blood cell, this interaction is lost. Hemoglobin has high oxygen affinity and thus poor oxygen-releasing characteristics. (3) Inside the red blood cell, hemoglobin does not contribute to plasma oncotic pressure. However, if the total hemoglobin concentration in whole blood (~14 g/dl) was free in solution, it would exert an oncotic pressure twice that found in the circulation. Thus, a lower hemoglobin concentration would have to be used for infusion.

The main problems associated with hemoglobin namely (i) intravascular dimerization, (ii) high oxygen affinity, and (iii) oncotic pressure restrictions have been addressed by a variety of chemical modifications. These chemical modifications have

the potential to satisfy different requirements. For example, pyridoxal-5-phosphate is analogous to 2,3 DPG. Benesch showed that pyridoxal 5'-phosphate, a bifunctional organic phosphate, has analogous effects to 2,3 DPG on lower oxygen affinity of hemoglobin [25]. Pyridoxylated hemoglobin has effective oxygenation characteristics, but dimerization is still a problem. Chemical modifications used to stabilize the tetramer form of hemoglobin or to produce hemoglobin polymers are described below.

1.2.3 Modified Hemoglobin

Intermolecular crosslinked hemoglobin

As first reported by Chang [15-17]. Intermolecular crosslinking or polymerization is the process by which individual hemoglobin molecules are linked together to form polymers. Polymerization produces a variety of molecular weight sizes with intramolecular and intermolecular crosslinks. The non-specificity of polymerization and the difficulty in controlling rate of reaction are disadvantages with this approach. The dialdehyde crosslinking agent, glutaraldehyde, has been studied extensively [2,5,7,26-28]. Glutaraldehyde can react with ε-amine groups of lysine residues, terminal α-amino groups, and sulfhydryl group of cysteines. Other dialdehyde molecules have been produced by opening the ring structures of sugars or nucleotides, such as o-raffinose or ATP respectively [29,30]. Intermolecular crosslinking of hemoglobin molecules solves the dimerization problem. Furthermore, by producing polymers and reducing the total number of molecules, the oncotic pressure problem is solved. Therefore, a polymerized

solution can be produced containing the normal whole blood concentration of 14 g/dl. Pyridoxylated polyhemoglobin has shown great promise as a red blood cell substitute. In fact, it was the first modified hemoglobin tested clinically [31].

Intramolecular crosslinked hemoglobin

Intramolecular crosslinking of hemoglobin is directed at stabilizing the tetrameric conformation, thus preventing dimer formation and rapid renal excretion. Intramolecular crosslinkers include DBBF (bis(3,5-dibromosalicyl)fumarate), and NFPLP (nor-2-formylpyridoxl-5'-phosphate). DBBF crosslinks hemoglobin between the α-99 lysine residues. NFPLP crosslinks hemoglobin between the β1 and β182 amino acid residues. Both modifications have the dual effects of tetramer stabilization and lowering oxygen affinity [2,5,7,18,19]. DBBF can also be used to crosslink the β chains. With intramolecular modification, the hemoglobin concentration used would have to be lower than physiological values due to oncotic pressure considerations.

Conjugated hemoglobin

In 1964, Chang crosslinked hemoglobin to macromolecules, such as polyamide and nylon, producing solid microspheres of conjugated hemoglobin [15-17]. Subsequently, hemoglobin was crosslinked to soluble macromolecules producing soluble hemoglobin conjugates with increased circulation time. Some approaches include the use of dextran, polyethylene glycol, and polyoxyethylene [32-34].

Additional oxygen affinity modifications must be performed in the case of human hemoglobin.

Microencapsulated hemoglobin

In 1957, Chang first reported the use of artificial red blood cells [14,15] This approach replaces red blood cell membrane with a synthetic ultrathin membrane which retains hemoglobin inside the artificial cells. Hemoglobin retains functional activity within the artificial cell. Furthermore, important red blood cell cofactors or enzymes can be included within these artificial cells. The synthetic membrane does not contain red cell antigens, thus eliminating the need for compatibility testing. Since Chang's pioneering work, he and others have explored different hemoglobin microencapsulation techniques. Specifically, different membrane materials have been used (eg. lipid vesicles) to produce smaller structures with increased circulation times [2,5,7]. Recent interest has focused on the use of polylactic acids and polyglycolides. These compounds can be used to produce biodegradable polymer membrane nanocapsules containing hemoglobin [35].

Alternate sources of hemoglobin

Human hemoglobin can be produced using recombinant DNA technology. Studies have been performed using bacteria (*Escherichia coli*) [36] and yeast (*Saccharomyces cerevisiae)* [37]. Expression of human hemoglobin has also been achieved in transgenic pigs and mice [38,39]. In pigs, levels of human hemoglobin are present in excess of

15% of the total cellular hemoglobin. In mice, greater than 80% of hemoglobin is human. Recombinant hemoglobin still suffers from the limitations outlined in section 1.2.2 unless chemical modifications are incorporated. However, it is possible to overcome these limitations by using site-directed mutagenesis. A recombinant hemoglobin has been produced using *E. coli* that has extended half-life and good oxygen dissociation characteristics [40]. This product is now being used in clinical trials. The greatest challenge in recombinant hemoglobin technology is achieving large scale production at minimum cost. It must be emphasized that although recombinant hemoglobin may eventually represent a useful source of raw material, it still faces the same safety issues as current hemoglobin substitutes. Unless, of course, additional genetic modifications can manage to reduce this toxicity.

An alternative source of raw material is bovine hemoglobin. The oxygen affinity of human hemoglobin depends on the interaction with 2,3 DPG in the red blood cell. Bovine hemoglobin differs from human hemoglobin in that chloride anion modulates oxygen affinity as opposed to 2,3 DPG [41,42]. Therefore, bovine hemoglobin has a naturally lower oxygen affinity than human hemoglobin, and thus the only modification necessary is a means of stabilizing the tetramer. The large scale availability and absence of human viruses are also potential advantages with bovine hemoglobin. There may be disadvantages with the use of bovine hemoglobin namely the potential transmission of bovine spongiform encephalitis, allergic response to large infusions of a foreign protein, and antibody formation upon repeat infusions of bovine protein in humans.

Clinical trials of modified hemoglobin

Savitsky's trials with purified "stroma-free" hemoglobin suggested that even purified hemoglobin could induce renal toxicity [23]. Moss and his colleagues postulated that hemoglobin nephrotoxicity was due to the filtering of hemoglobin dimers. Moss and his group were the first to perform clinical trials with modified hemoglobin [31]. They infused 0.26 g/kg of glutaraldehyde-polymerized pyridoxylated hemoglobin into six healthy volunteers. The authors did not observe any renal dysfunction or coagulation defects, however one recipient experienced an allergic response. Subsequent studies with glutaraldehyde-polymerized pyridoxylated hemoglobin using doses of 0.125-0.6 g/kg revealed no adverse effects. Similar large dosage studies have also been carried out in trauma and surgical patients with apparently no major adverse effects reported. They have now infused up to three liters per patient in clinical trials. In 1991, Garcia-Gallont reported their safety trials with glutaraldehyde-polymerized bovine hemoglobin [43]. They infused approximately the same dosage as the initial study by Moss, and found no adverse effects. However, similar studies performed in the United States were terminated due to unreported events [18]. Since then, glutaraldehyde-polymerized bovine hemoglobin is once more being tested clinically. Another polymerized bovine hemoglobin was used successfully to treat patients suffering from sickle cell crisis [44]. A genetically engineered hemoglobin has also been clinically tested at doses of 0.015-0.320 g/kg in healthy volunteers [19]. At higher doses, there was an increase in blood pressure and bradycardia. Patients also

suffered from vomiting, nausea, and abdominal pain. Diaspirin crosslinked hemoglobin is also under clinical evaluation. Initial studies in normal volunteers showed bradycardia and an increase in blood pressure. No other effects were reported. Clinical trials of several modified hemoglobins are still ongoing with emphasis on safety issues. Unfortunately, the results of clinical trials are not always made public.

1.3 Pro-oxidant Potential of Hemoglobin and Modified Hemoglobin

1.3.1 Hemoglobin and Free Radicals

Reactive oxygen species in vivo

The addition of one electron or univalent reduction of molecular oxygen generates the superoxide anion radical or simply superoxide ($O_2^{\bullet-}$). The two electron reduction product of oxygen is hydrogen peroxide (H_2O_2). By definition, hydrogen peroxide is not a free radical since it does not contain unpaired electrons. Hydrogen peroxide, however, is an important oxidant in biological systems, and therefore belongs in the class of reactive oxygen species. The reduction of oxygen by three electrons generates hydroxyl radical ($^{\bullet}OH$). Hydroxyl radical is a highly reactive species. It has an extremely short half-life, and can damage a variety of biological molecules within a small radius of its site of production [45,46]. Superoxide spontaneously converts to hydrogen peroxide at physiologic pH, this reaction is considerably accelerated by superoxide dismutase. Superoxide can spontaneously interact with hydrogen peroxide to generate hydroxyl radical, but this reaction is very slow at physiological pH (Haber-Weiss reaction).

However, the rate of this reaction can be significantly increased in the presence of transition metals. Hydrogen peroxide reacts with ferrous (Fe^{2+}) iron to generate hydroxyl radical (Fenton reaction). An important dual effect of superoxide is that it can reduce ferric iron and act as a source of hydrogen peroxide. Thus, hydroxyl radical can be generated by a reaction scheme termed the iron-catalyzed Haber-Weiss reaction or superoxide-driven Fenton reaction (Figure 1.1) [45,46]. Another non-iron dependent mechanism of hydroxyl radical formation that has been suggested involves the reaction of superoxide with nitric oxide (NO). This reaction produces peroxynitrite ($OONO^-$) which at physiological pH may decompose to yield hydroxyl radical and nitrogen dioxide [47].

Reactive oxygen species are formed via several mechanisms in vivo. Reactive oxygen species are generated as byproducts of aerobic metabolism. During oxidative phosphorylation, mitochondrial respiratory chain catalyzes the four electron reduction of oxygen to water generating adenosine triphosphate (ATP). However, partially reduced forms of oxygen may be liberated from the respiratory chain during the tetravalent reduction of oxygen. Conditions, such as ischemia or high oxygen tension, increase the leakage of free radicals from respiratory chain enzymes [48,49].

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

$$O_2^{\cdot-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad (2)$$

$$\underline{H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH} \qquad (3)$$

$$H_2O_2 + O_2^{\cdot-} \rightarrow OH^- + {}^{\cdot}OH + O_2 \qquad (4)$$

Figure 1.1: Iron-catalyzed formation of hydroxyl radical. Reaction (1) occurs spontaneously or via by superoxide dismutase, and supplies hydrogen peroxide for reaction (3). Superoxide acts as a reductant for ferric iron, generating ferrous iron for reaction (3). The net result of reaction (2) and (3) is reaction (4) which is termed the iron-catalyzed Haber-Weiss or superoxide-driven Fenton reaction.

Activated polymorphonuclear leukocytes possess a plasma membrane-associated NADPH oxidase which generates superoxide radical by catalyzing the reduction of molecular oxygen. This enzyme enables these cells to destroy bacteria and other foreign material with a variety of oxidizing agents that, in addition to superoxide, include hydrogen peroxide, hypochlorous acid (HOCl), and N-substituted chloramines (R-NHCl) [50]. Hypochlorous acid is produced by the action of myeloperoxidase which catalyzes the reaction of hydrogen peroxide and chloride ions. This latter enzyme depends on NADPH oxidase to supply superoxide which can then dismutate to hydrogen peroxide. The importance of this function is emphasized in patients with

chronic granulomatous disease. In these patients, neutrophils are genetically deficient in NADPH oxidase, and thus these patients are unable to destroy commonly encountered microorganisms. NADPH oxidase is also found in other phagocytic cells such as macrophages and monocytes. Another important free radical source is xanthine oxidase. This enzyme catalyzes the reduction of oxygen to superoxide during the two-step breakdown of hypoxanthine to uric acid. Xanthine oxidase activity is especially relevant in the context of ischemia-reperfusion injury [51,52]. The metabolism of arachidonic acid by cyclooxygenase and lipooxygenase to produce prostaglandins and leukotrienes respectively involves the formation of peroxy radicals [52].

In biological systems, reactive oxygen species react with lipids, proteins, nucleic acids, and carbohydrates. Single and double strand breaks in DNA and base hydroxylation occurs following oxidative attack on DNA. Repeated free radical attack on DNA may be related to increased mutation of genetic material [45,46]. Reactive free radicals can modify amino acid residues of proteins and lead to crosslinking, changes in conformation, and loss of function due to site-specific damage at active sites [53,54]. Both intracellular and extracellular proteins are critical targets for free radical attack.

A significant mechanism of free radical damage is the peroxidation of polyunsaturated fatty acids [55]. This lipid peroxidation process occurs via a classical chain reaction mechanism (Figure 1.2). By definition, polyunsaturated fatty acids contain two or more double bonds. Methylene hydrogens located between two adjacent double bonds are highly susceptible to free radical attack. The initiating free radical

attack on polyunsaturated fatty acids (RH) abstracts a methylene hydrogen and generates an organic free radical ($R^{\bullet}$). Following electron rearrangement into a diene conjugate, $R^{\bullet}$ rapidly adds oxygen to form a peroxyl radical ($ROO^{\bullet}$). Peroxyl radicals propagate a chain reaction process by reacting with another PUFA. Hydrogen abstraction by peroxyl radicals produces lipid hydroperoxides. Lipid hydroperoxides can themselves decompose and generate highly reactive species capable of initiating further peroxidation events.

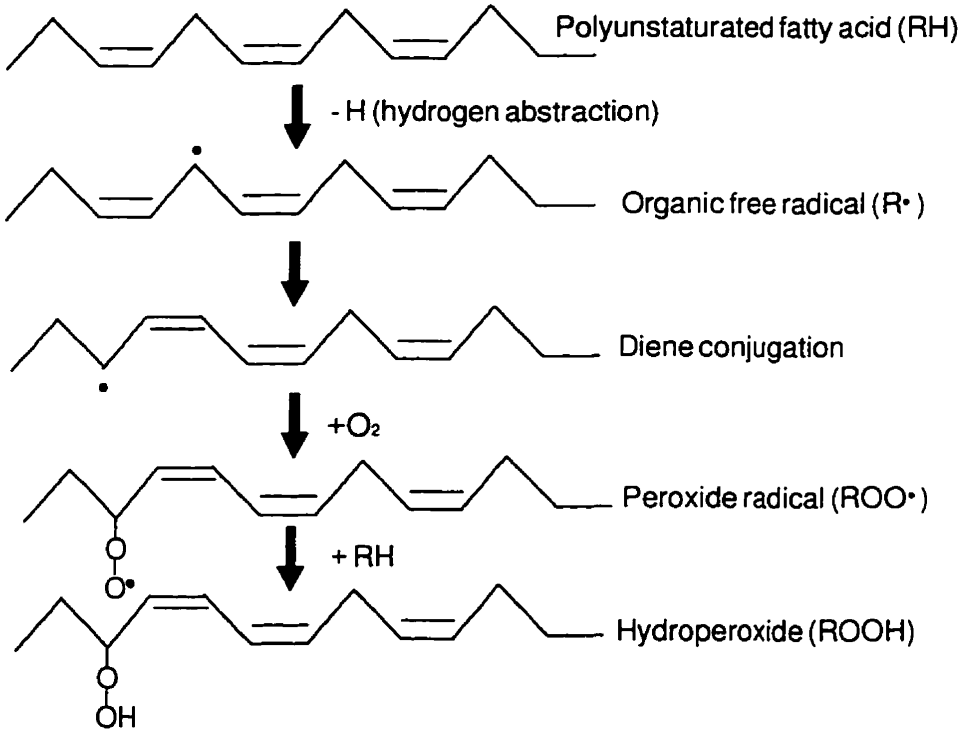

Figure 1.2: Schematic representation of lipid peroxidation mechanism.

The potential role of reactive oxygen species in various human disease states has led to an increased need for methodologies aimed at measuring free radicals and their reactions in vivo. However, direct measurement of free radicals in vivo is problematic due to the highly reactive nature of these species. Methods have focused primarily on measuring the products of free radical attack such as lipid peroxidation (conjugated dienes, MDA, lipid hydroperoxides), protein carbonyls, and oxidized DNA bases. Electron paramagnetic spectroscopy (EPR) and spin trapping techniques are used to directly measure in vivo free radical generation [56,57]. This technique is based on the reaction of free radicals with spin trapping agents to produce more stable compounds that can be detected by EPR. Another technique based on the hydroxylation of aromatic compounds has also received considerable attention. This assay provides an indirect measure of hydroxyl radical by analyzing the products resulting from their attack on aromatic compounds, such as salicylate, phenylanaline, and 4-hydroxybenzoate [58,59]. This method has been used to study hydroxyl radical formation in heart, brain, intestine, eye, lung, diaphragm, skeletal muscle and whole body [60-70].

Hemoglobin and free radical reactions

Hemoglobin can promote the production of free radicals by several mechanisms. Spontaneous autoxidation of hemoglobin (Fe^{2+}-heme) to methemoglobin (Fe^{3+}-heme) generates superoxide [71,72]. About 1-3 % of hemoglobin in red blood cells is present as non-oxygen binding methemoglobin. Superoxide can produce hydrogen peroxide.

Both these species may further oxidize hemoglobin or other red cell constituents. Fortunately, these reactive species are detoxified by a host of antioxidant defense systems in the red blood cell. In addition, the red blood cell contains a methemoglobin reductase enzyme system that prevents the accumulation of methemoglobin by reconverting it back to the functional ferrous form. However, outside the red blood cell, hemoglobin is not subject to similar regenerative mechanisms or antioxidant protection. Acellular hemoglobin, therefore, is highly susceptible to interaction with oxidants generated from its own autoxidation, and from other endogenously produced oxidants. Furthermore, methemoglobin releases heme more readily than its ferrous counterpart increasing the potential for iron-mediated free radical toxicity [73].

Excessive oxidative damage to hemoglobin leads to the release of iron from heme. In the presence of free iron, superoxide and hydrogen peroxide rapidly react to produce hydroxyl radical [74,75]. Hydrogen peroxide can also react with hemoglobin to produce ferrylhemoglobin ($^{\bullet}HbFe^{IV}=O$ / $HbFe^{IV}=O$) [76,77]. Ferrylhemoglobin is a highly reactive species that can induce lipid peroxidation and oxidize other biological molecules [78]. It may represent a more physiologically relevant mechanism of hemoglobin free radical toxicity since it does not depend on the release of iron from hemoglobin. Hemoglobin and modified hemoglobins have also been shown to activate phagocytic respiratory burst mechanisms. Murine macrophages and human monocytes increased their production of hydrogen peroxide and lipid peroxides following exposure to native and modified bovine hemoglobins [79].

Another important free radical interaction involves the reaction of hemoglobin with nitric oxide (NO). Nitric oxide has been identified as the endothelium-derived relaxing factor [80]. The scavenging of NO by hemoglobin is thought to be partly responsible for the vasoconstrictive effect observed clinically and experimentally with some modified hemoglobins [81-83]. Nitric oxide is produced by endothelial cells via NO synthase. This enzyme catalyzes the oxidation of L-arginine in the presence of NADPH to nitric oxide and citrulline. Nitric oxide diffuses to smooth muscle cells where it activates guanylate cyclase. This latter enzyme activates the production of cyclic GMP (cGMP) which subsequently induces vasodilation by stimulating the binding of intracellular free calcium. It has also been shown that superoxide can inactivate NO, and cause vasoconstriction [84].

The different propensity of modified hemoglobins to undergo oxidative reactions appears to be related to the nature of hemoglobin modification [85-88]. Studies suggest that the stabilization of β-chain of hemoglobin reduces oxidative reactivity of hemoglobin. Intramolecularly crosslinked hemoglobin, linked between the α chains, was more likely to form ferrylhemoglobin upon hydrogen peroxide exposure than native hemoglobin. Whereas, β-crosslinked hemoglobin exhibited less ferryl radical upon similar oxidant challenge [87]. In another study, modified bovine hemoglobin intramolecularly crosslinked between the β-chains, showed less autoxidation and oxidant interaction than intermolecularly glutaraldehyde-linked bovine hemoglobin

[88]. It has been shown that glutaraldehyde modification increases autoxidation rate of hemoglobin [89].

Hemoglobin and free radical-mediated damage

The ability of hemoglobin to induce lipid peroxidation has long been recognized [90-94]. Faassen et al showed the increased peroxidation of murine brain homogenates following their incubation with hemoglobin [94]. This process was attributed to the release of iron from hemoglobin since transferrin and deferoxamine inhibited peroxidation. The authors of this study also suggested that antioxidant addition to modified hemoglobins may provide some protective benefits. Endothelial cells are highly susceptible to cytotoxic effects of hemoglobin [95-98]. The release of heme from methemoglobin induces endothelial damage [96,97]. Activated polymorphonuclear cells potentiate this effect by oxidizing hemoglobin to methemoglobin [96]. In this context, hemoglobin and modified hemoglobin have also been shown to stimulate NADPH oxidase activity of phagocytes [79]. This effect may also be important in the context of ischemia-reperfusion syndromes since neutrophil infiltration and activation plays an important role in the pathogenesis of reperfusion injury [99]. Alternatively, it was shown that ferrylhemoglobin (Fe^{4+}) produces greater cellular injury than methemoglobin (Fe^{3+}) or ferrohemoglobin (Fe^{2+}) in human umbilical vein endothelial cells [98]. Another relevant finding is that endothelial cells subject to periods of

hypoxia-reoxygenation increase their release of superoxide and hydrogen peroxide [100].

Several studies suggest that in vivo toxicity of hemoglobin is partly mediated via free radical processes. Histological changes in swine liver and kidney following exchange transfusion with α-crosslinked bis(3,5 dibrosalicyl)fumarate-Hb (DBBF-Hb) are indicative of possible lipid peroxidation events [101]. Exchange transfusion with modified bovine hemoglobin increased lipid peroxidation in rat heart, liver and plasma [102]. In another study, the nephrotoxicity induced by hemoglobin in rats was reduced with the iron chelator, deferoxamine, suggesting an iron-mediated mechanism of damage [103]. In the same study, hemoglobin infusion induced greater renal dysfunction in ischemic kidneys compared to non-ischemic kidneys. Hemoglobin neurotoxicity has also been attributed to heme- and iron-mediated oxidative mechanisms [104]. Resuscitation of hemorrhaged dogs with stroma-free hemoglobin is associated with increased formation of salicylate hydroxylation products, indicative of hydroxyl radical formation [105]. In another study, it was shown that deferoxamine reversed the vasoconstrictive effect of a modified hemoglobin presumably by inhibiting iron-mediated free radical process [83].

Special case of ischemia-reperfusion injury

Ischemia can be defined as insufficient oxygen supply to meet the metabolic demands of the tissue. The restoration of normal oxygen supply is required to prevent

ensuing cellular damage and death. Paradoxically, the return of oxygen under certain circumstances can aggravate injury or dysfunction. This injury component is termed reperfusion injury, and it is distinct from ischemic injury. In 1981, Granger and his coworkers performed the initial studies of ischemia-reperfusion injury in feline small intestine [106]. They showed that the administration of superoxide dismutase near the end of ischemia, but before reperfusion, largely prevented post-ischemic injury of the microvasculature. Protection was also noted when the enzyme xanthine oxidase was inhibited with allopurinol. This suggested that the injury occurred at reperfusion, and that it was mediated by xanthine oxidase-generated superoxide radical. Xanthine oxidoreducutase catalyzes the oxidation of purines to uric acid. The enzyme exists in two active forms. The xanthine dehydrogenase form uses NAD^+ as its electron acceptor, and thus generates NADH. The xanthine oxidase form uses oxygen as its electron acceptor, and thus produces superoxide radical. Xanthine dehydrogenase normally predominates in tissue, however it can be converted to xanthine oxidase by limited proteolysis or sulfhydryl group oxidation. Based on these observations, a mechanism was proposed to account for ischemia-reperfusion injury. This scheme is based on two main events, (1) ischemia results in the accumulation of xanthine/hypoxanthine due to degradation of ATP stores, and (2) xanthine dehydrogenase is converted to xanthine oxidase. The return of oxygen with reperfusion is used by xanthine oxidase to oxidize purines and generate superoxide radical. The findings that hydroxyl radical scavengers or iron chelators are effective in reducing postischemic tissue dysfunction suggest

secondarily generated hydroxyl radical contributes to reperfusion injury [107-109]. Intestine is a particularly rich source of xanthine dehydrogenase/oxidase, and is known to be particularly vulnerable to ischemia-reperfusion injury [51]. Some evidence suggests oxidant-injured cells may release xanthine oxidase into systemic circulation and mediate damaging effects at distant sites from original injury [110,111]

The endothelial trigger mechanism was proposed following studies performed with rat pulmonary artery endothelial cells subject to periods of hypoxia-reoxygenation (simulated ischemia-reperfusion) [112]. It was found that xanthine dehydrogenase converted to xanthine oxidase during ischemia, and this was followed by endothelial cell lysis at reoxygenation [112]. Cell lysis was prevented in the presence of allopurinol or superoxide dismutase and catalase at reoxygenation. Thus, the initiating steps of reperfusion injury were proposed to occur in the endothelium with the production of superoxide radical. The infiltration and activation of neutrophils to the site of injury is also thought to be a major contributor to reperfusion injury [113]. Reactive oxygen species may also attract and activate inflammatory phagocytes which can themselves induce injury by the release of cytotoxic mediators. Studies have shown the reaction of superoxide radical with plasma produces chemotactic stimuli for neutrophils [114]. Altered endogenous antioxidant mechanisms following ischemia may also contribute to increased oxidative stress [115].

Free radical mechanisms are also thought to play an important role in skeletal muscle reperfusion injury. Studies have shown the detection of reactive oxygen species

by EPR spectroscopy, enhanced lipid peroxidation (malondialdehyde, lipid hydroperoxides, conjugated dienes), increased xanthine dehydrogenase conversion to xanthine oxidase, accumulation of end-purine metabolites, neutrophil infiltration and activation during reperfusion [116-120]. Other findings have shown that reperfusion effects were reduced with free radical scavengers (SOD, catalase, dimethylthiourea), iron chelators (deferoxamine), and inhibitors of free radical production (allopurinol) [107, 121-125]. It has been shown that xanthine oxidase levels increase significantly following a two hour period of ischemia, whereas shorter ischemia times do not induce significant increase in xanthine oxidase [107]. Iron sequestered in endogenous sites, like ferritin, can be released by reacting with superoxide radical which reduces the ferric iron of ferritin [126]. The release of myoglobin from damaged tissues may also be implicated in heme- or iron-mediated postischemic injury [45,127]. Clinical implications for skeletal muscle ischemia-reperfusion injury include; surgical revascularization of the acutely ischemic limb, prolonged use of the tourniquet during orthopedic surgery, and aorta cross-clamping procedures [128, 129].

Reperfusion injury is attributed to free radicals produced following the return of oxygen to ischemic tissues. Modified hemoglobins used to deliver oxygen under these oxidative stress conditions may further promote and exacerbate oxidant tissue damage. In this context, a desirable oxygen carrier may be one that delivers oxygen effectively, and at the same time prevents the increase in oxygen-derived free radicals and/or ameliorates pre-existing oxidative stress.

1.3.2 Endogenous Antioxidant Mechanisms

Overview

Endogenous oxidants have damaging effects as well as physiologically beneficial ones. Endogenous antioxidants provide important defense mechanisms that allow maintenance of an oxidant balance. In many pathophysiological situations, the production of free radicals exceeds local endogenous antioxidant capabilities, and this results in tissue injury. These antioxidant defense mechanisms can be categorized as follows; enzymatic detoxification of primary radical species generated from molecular oxygen, iron sequestration systems preventing iron-catalyzed free radical formation, and detoxification of free radical species produced secondarily due to free radical attack.

Superoxide dismutase and catalase

Superoxide dismutases are metal-containing enzymes that catalyze the reduction of superoxide radical to hydrogen peroxide and oxygen [130]. Three forms of SOD have been described in mammals. There is an intracellular SOD which contains copper/zinc (Cu/Zn) in its active site, and is present in the cytoplasm. The second form, maganese (Mn)-SOD is present in high concentrations in the mitochondria. The third form is an extracellular (EC)-SOD which also contains copper and zinc at its active site. Bovine erythrocyte SOD is among the class of intracellular Cu/Zn-containing SODs. The intracellular Cu/Zn form has a molecular weight of 32 kD, and consists of two identical subunits. Each subunit contains one Cu and one Zn atom. The Cu atom

functions in the reaction by undergoing oxidation and reduction, whereas Zn is thought to stabilize the active site [131,132]. (Figure 1.3)

Catalase is an intracellular heme-containing enzyme that catalyzes the breakdown of hydrogen peroxide to water and oxygen [132]. Catalase is usually present in peroxisomes, whereas in red blood cells it is in the cytosol. High levels of catalase are found in red blood cells and liver, whereas low levels are present in heart, brain, and skeletal muscle. Mammalian catalase has a molecular weight of approximately 232 kD, and is composed of four identical subunits. Each subunit contains a ferric-heme active site. One molecule of NADPH bound to each subunit stabilizes the active site (Figure 1.3).

Superoxide dismutase

$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$

Rxn. mechanism: $SOD\text{-}Cu^{2+} + O_2^{\bullet-} \rightarrow SOD\text{-}Cu^{+} + O_2$

$SOD\text{-}Cu^{+} + O_2^{\bullet-} + 2H^+ \rightarrow SOD\text{-}Cu^{2+} + H_2O_2$

Catalase

$2H_2O_2 \rightarrow 2H_2O + O_2$

Rxn. mechanism: $Catalase\text{-}Fe^{3+} + H_2O_2 \rightarrow Catalase\text{-}[Fe^{4+}{=}O]^{\bullet}$

$Catalase\text{-}[Fe^{4+}{=}O]^{\bullet} + H_2O_2 \rightarrow Catalase\text{-}Fe^{3+} + H_2O + \frac{1}{2}\, O_2$

Figure 1.3: Reaction mechanisms of superoxide dismutase and catalase.

Therapeutic interventions based on SOD and/or catalase have been extensively explored as potential treatments against free radical-mediated disease states, such as

reperfusion injury [133]. The main disadvantage is the short circulation time of superoxide dismutase and catalase. Several approaches are aimed at increasing intravascular persistence of these enzymes for example, polyethylene glycol conjugation and liposome encapsulation. Another important issue is the bell-shaped dose response curve observed for superoxide dismutase. It has been shown that low doses of SOD protects hearts from post-ischemic injury, whereas high doses of SOD afforded no protection or exacerbated injury [134].

Normal physiological mechanisms provide strong conceptual support for combining hemoglobin, SOD, and catalase. Red blood cells contain high amounts of superoxide dismutase, catalase, and other antioxidants. These antioxidants help maintain the functional ferrous form of hemoglobin, and protect hemoglobin and other red cell constituents from oxidative reactions. During the preparation of modified hemoglobin solutions, extensive purification of hemoglobin must be used in order to avoid the toxicity of red blood cell contaminants. Thus, purified hemoglobin is devoid of these protective antioxidant defenses.

Other enzyme and non-enzyme antioxidants

Glutathione peroxidase is a selenium-containing enzyme composed of four subunits, and is present in the cytosol [52,132]. This peroxidase detoxifies hydrogen peroxide and lipid hydroperoxides by oxidizing two molecules of reduced glutathione to glutathione disulfide. Reduced glutathione is regenerated by the action of glutathione

reductase in the presence of NADPH. This NADPH is subsequently regenerated by the pentose phosphate pathway. Another non-selenium glutathione peroxidase catalyzes the reduction of lipid hydroperoxides only.

Iron-binding or sequestering systems may also be considered antioxidant defense mechanisms since they prevent iron-mediated free radical processes. Plasma haptoglobin and hemopexin bind hemoglobin dimers and heme respectively. Ferritin acts as an intracellular iron storage protein, and transferrin binds iron in the plasma. The action of these proteins helps prevent free iron from participating in the Fenton reaction [132].

Small water soluble molecules such as reduced ascorbic acid scavenges superoxide and hydroxyl radical, and can inhibit lipid peroxidation at high concentrations. Whereas, low concentrations of ascorbic acid can promote lipid peroxidation in the presence of iron. Vitamin E (α-tocopherol) prevents the propagation phase of lipid peroxidation. Vitamin E is a lipid-soluble antioxidant which reacts with lipid peroxyl radicals to form the tocopheryl radical. This species can be converted back to reduced form by ascorbic acid. β-carotene is also efficient scavenger of lipid peroxyl radicals, and may be especially important in situations when vitamin E stores are depleted.

1.4 Aim of Study

Crosslinked Hemoglobin - Superoxide Dismutase - Catalase: A Novel Red Blood Cell Substitute Designed To Limit Free Radical-Mediated Toxicity

1.4.1 Main Objective

The pro-oxidant activity of hemoglobin is a critical safety issue associated with modified hemoglobin-based red blood cell substitutes. Considerable interest focuses on the development of potentially safer modified hemoglobin oxygen carriers that possess reduced free radical reactivity. Hemoglobin can generate or participate in the formation of harmful reactive oxygen species, and promote free radical-mediated toxicity in vivo. These pro-oxidant reactions directly or indirectly involve superoxide radical and hydrogen peroxide. Superoxide dismutase and catalase are endogenous antioxidant enzymes that catalyze the breakdown of superoxide radical and hydrogen peroxide respectively. This research introduces a novel approach based on intermolecularly crosslinking hemoglobin, superoxide dismutase, and catalase. The combination of hemoglobin with these two antioxidants is designed to produce a type of modified hemoglobin with reduced free radical reactivity [135].

1.4.2 Rationale For Thesis Research

My thesis research can be divided into three main phases. The first phase involved studying the preparation, structural and functional properties of crosslinked

hemoglobin, superoxide dismutase, and catalase (PolyHb-SOD-CAT). Glutaraldehyde, a non-specific dialdehyde crosslinker, is used to polymerize hemoglobin and trace amounts of superoxide dismutase and catalase. This process produces a heterogeneous mixture of molecules varying in size and protein composition. Intermolecular crosslinking is designed to overcome problems associated with the short circulation times of free hemoglobin, SOD, and catalase.

In the second phase, I study the effects of in vitro oxidant exposure on PolyHb and PolyHb-SOD-CAT. The interaction of oxidants with hemoglobin produces highly reactive secondary radicals, ferrylhemoglobin and hydroxyl radical. These species mediate direct tissue damage. One potential role of PolyHb-SOD-CAT is its ability to prevent the formation of these species during oxidant exposure. Therefore, I study the formation of ferrylhemoglobin and hydroxyl radical following in vitro oxidant challenge with superoxide and hydrogen peroxide. I also assess in vitro oxidative damage by analyzing lipid peroxidation of phospholipid liposomes.

In the third phase, I study the effects of PolyHb-SOD-CAT in models of in vivo oxidative stress. The role of modified hemoglobins in ischemia-reperfusion injury is a specific source of concern (Figure 1.4). The return of oxygen to tissues or organs following a period of ischemia is associated with a burst of free radical generation. This increased oxidative stress is thought to mediate reperfusion injury. The use of modified hemoglobin oxygen carriers in these situations may actually aggravate oxidative stress and injury. Two models of ischemia-reperfusion injury are studied, the isolated rat

hindlimb and the isolated rat intestine. In both models, the formation of hydroxyl radical during reperfusion with PolyHb-SOD-CAT is compared to PolyHb.

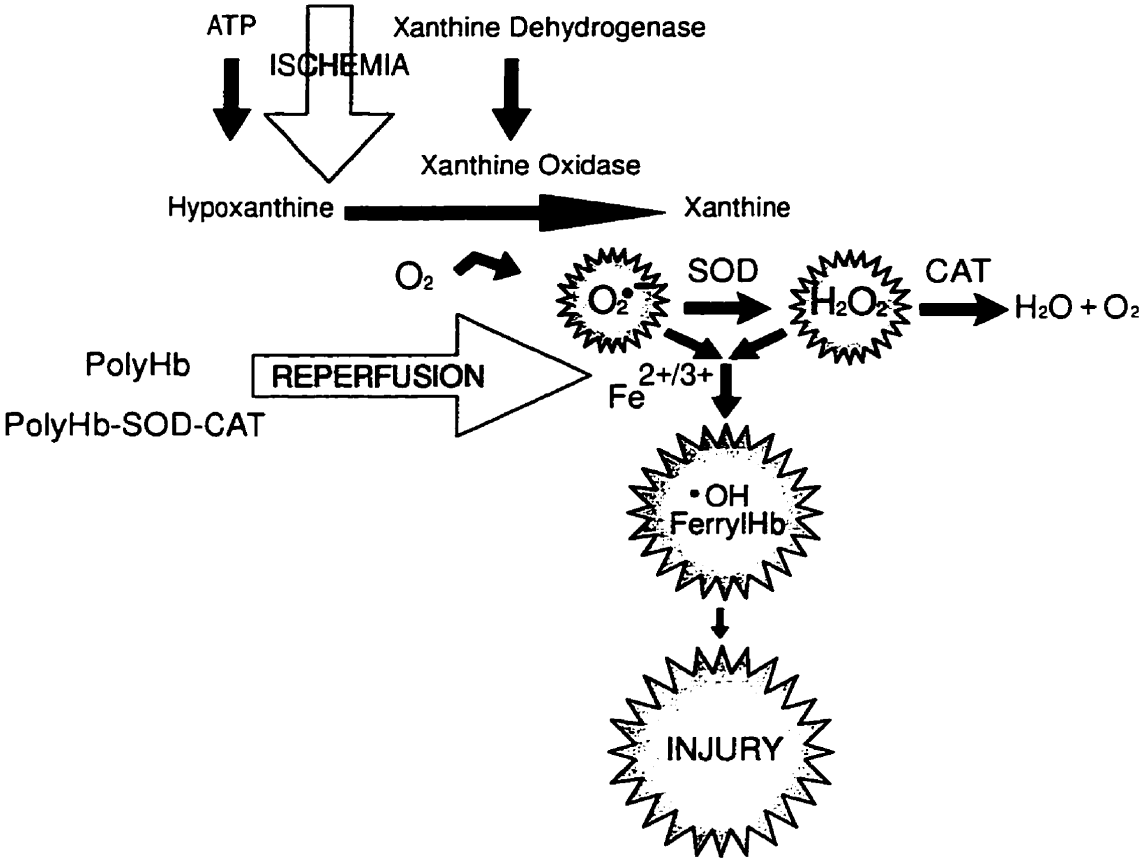

Figure 1.4: Schematic diagram of the potential role of modified hemoglobin in ischemia-reperfusion injury. Ischemia causes the accumulation of purines and the conversion of xanthine dehydrogenase to xanthine oxidase. The return of oxygen initiates the production of superoxide radical which via a series of reactions ultimately leads to tissue injury. Reperfusion with polymerized hemoglobin (PolyHb) may aggravate situation by increasing production of free radicals and supplying heme or free iron. PolyHb-SOD-CAT, however, combines oxygen delivery with the elimination of superoxide and hydrogen peroxide by SOD and catalase respectively.

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CHAPTER 2

Crosslinked Hemoglobin-Superoxide Dismutase-Catalase Scavenges Oxygen-Derived Free Radicals and Prevents Methemoglobin Formation and Iron Release

2.1 Purpose of Chapter

Chapter 2 presents the first series of experiments I conducted in evaluating the feasibility and potential benefits associated with crosslinking hemoglobin, superoxide dismutase, and catalase (PolyHb-SOD-CAT). The primary focus was to determine whether superoxide dismutase and catalase would retain their respective antioxidant functions following glutaraldehyde polymerization. Another important objective was to show that superoxide dismutase and catalase could protect hemoglobin from oxidative reactions and degradation. The results with PolyHb-SOD-CAT were compared with polymerized hemoglobin (PolyHb) which served as the control. The promising results obtained in this chapter set the stage for several other research directions that subsequent chapters tried to address in some form. Furthermore, during the course of this research it was frequently necessary to manipulate assay techniques in order to account for assay interference due to hemoglobin. This experience proved useful in subsequent experimentation where I adapted similar techniques for different purposes or in developing entirely new methodologies.

2.2 Abstract

In this study, we prepared PolyHb-SOD-CAT (intermolecularly crosslinked hemoglobin, superoxide dismutase and catalase). We found that PolyHb-SOD-CAT is effective in scavenging oxygen-derived free radicals. In the xanthine/xanthine oxidase system, the initial rate of cytochrome c reduction was 2.13 ± 0.26 nmoles cyt. c/min for PolyHb alone. PolyHb-SOD-CAT reduced this to 0.56 ± 0.08 nmoles cyt.c/min because of its ability to eliminate superoxide ($O_2^{\bullet-}$). Addition of PolyHb to 200 μM of hydrogen peroxide (H_2O_2), changed the H_2O_2 level slightly to 192 ± 0.4 μM. Addition of PolyHb-SOD-CAT, on the other hand, lower the level to 41 ± 0.3 μM. Results also show that both effects were dependent on the concentration of SOD and catalase crosslinked with hemoglobin. Oxidative challenge with H_2O_2 resulted in minimal changes in the absorbance spectra of PolyHb-SOD-CAT. With PolyHb, there were spectral changes reflecting the formation of methemoglobin and heme degradation. Furthermore, the amount of iron released, after incubation with 250 μM H_2O_2, was 6.8 ± 1.8 μg/dl for PolyHb-SOD-CAT and 76.6 ± 1.0 μg/dl for PolyHb. These results show that crosslinked SOD-CAT prevents oxidative reactions involving the hemoglobin component of PolyHb-SOD-CAT.

2.3 Introduction

The development of an acellular hemoglobin-based oxygen carrier presents several advantages over normal red blood cells [1-3]. Polymerized hemoglobin solutions have effective oxygen delivery characteristics, and are among the most widely studied [1-3]. However, they also have the potential to promote oxygen-derived free radical processes with possible adverse effects [3,4]. This can occur by various mechanisms; 1) in the presence of superoxide and hydrogen peroxide, heme iron (bound and/or released) can drive Fenton reactions generating highly reactive perferryl or hydroxyl radicals [5]; 2) heme groups can autoxidize and release superoxide [6]; and 3) modified hemoglobin solutions may stimulate phagocyte respiratory burst activity and increase arachidonic acid metabolism, leading to an increase in superoxide production [7].

Ischemia-reperfusion injury can occur following the reintroduction of oxygen to an ischemic tissue or organ, and is generally accepted to be mediated by oxygen-derived free radicals [8]. Thus, like other oxygen-delivering regimes (e.g. red blood cells), hemoglobin-based products may cause reperfusion injuries. A desirable oxygen carrier would be one that delivers oxygen effectively, and at the same time prevents the increase in oxygen-derived free radicals and/or ameliorated pre-existing oxidative stress.

Endogenous antioxidant enzymes, superoxide dismutase (SOD) and catalase, catalyze the breakdown of superoxide and hydrogen peroxide respectively. Although

not all results are consistent, many reports have shown that SOD and/or catalase-based interventions are effective in reducing reperfusion injury and other free radical-mediated injury processes [5,9,10].

In this report, we present an original approach, extending earlier observations from this laboratory [11]. In the present study, we chemically crosslinked SOD and catalase with hemoglobin to form PolyHb-SOD-CAT. These experiments investigate the oxygen-derived free radical scavenging activity of PolyHb-SOD-CAT in vitro, and determine whether crosslinked SOD-catalase protects crosslinked hemoglobin components from oxidative reactions.

2.4 Materials and methods

Materials

Xanthine oxidase (20 U/ml) and xanthine were obtained from ICN Biomedicals. Superoxide dismutase from bovine erythrocytes (EC 1.15.1.1, 3000 units/mg stated activity) and catalase from beef liver (EC 1.11.1.6, 65000 units/mg stated activity) were purchased from Boehringer Mannheim. Purified bovine hemoglobin was used. Cytochrome c from horse heart (type III), 4-aminoantipyrine, horseradish peroxidase type IV (EC 1.11.1.7), ferrozine (0.85%), and the iron standard (500 g/dl) were obtained from Sigma. The hemoglobin assay kit was purchased from StanBio Labs. All other reagents were of analytical grade.

Preparation of PolyHb and PolyHb-SOD-Catalase

Polyhemoglobin (PolyHb) was prepared as previously described [12]. PolyHb-SOD-catalase was prepared as follows. Bovine hemoglobin (110 mg/ml), SOD (2 mg/ml), and catalase (20 mg/ml) were mixed in 0.1 M sodium phosphate buffer, pH 7.6 with the final ratio (as mg/ml) of Hb : SOD : catalase of 55 : 0.5 : 0.25. Following the addition of an initial amount of lysine-HCL (0.12 ml of 1.3 M / g Hb), glutaraldehyde (0.5 ml of 0.5M / g Hb) was added to crosslink the protein mixture. The reaction was allowed to proceed for 1.5-2 hrs. before being stopped by addition of excess lysine (0.78 ml of 2.0 M / g Hb). The resulting mixture was dialyzed against Ringer's solution then filtered through a 0.2 μm Nalgene filter. The hemoglobin concentration

was measured. Molecular weight distribution analysis was performed using gel filtration chromatography on a Sephadex G-200 column equilibrated with 0.1 M Tris-HCl , pH 7.5.

Scavenging of superoxide

The method was slightly modified from previously described [13]. This is based on the reduction of cytochrome c by superoxide. Each reaction mixture (3 ml) contains xanthine (50 μM), cytochrome c (10 μM), and catalase (10 nM) in 50 mM potassium phosphate buffer containing 0.1 mM EDTA at pH 7.8. Free catalase was added to reaction mixtures to prevent interference resulting from the accumulation of H_2O_2 [13]. Each reaction mixture also contains either PolyHb (5 μM) or PolyHb-SOD-catalase (5 μM). Addition of 10 μl xanthine oxidase (4 U/ml) starts the reaction at 22 °C. The rate of cytochrome c reduction was monitored at 550 nm with a Perkin-Elmer Lambda 4B Spectrophotometer. The molar extinction coefficient used for reduced cytochrome c was $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ [14].

Scavenging of hydrogen peroxide

The method was slightly modified from the previously described one [15]. Reaction volumes (3 ml) containing the horseradish peroxidase / 4-aminoantipyrine /phenol reagent solution (1.2 ml), PolyHb or PolyHb-SOD-CAT (5 μM), water, and hydrogen peroxide were prepared. Identical mixtures containing additional water

instead of H_2O_2 served as blanks. After allowing the mixture to stand for 3 min at 22 °C, the absorbance at 505 nm was recorded. Hydrogen peroxide and the reagent solution participate in a peroxidase-catalyzed reaction to form a dye which can be measured at this wavelength.

Monitoring absorbance spectra following oxidative challenge

Hydrogen peroxide was added to PolyHb (10 μM) or PolyHb-SOD-CAT (10 μM), and the absorbance spectra (450-700 nm) were recorded over time.

Iron measurement

As previously described [16]. The first step was to incubate PolyHb (15 μM) or PolyHb-SOD-CAT (15 μM) in hydrogen peroxide (total volume; 0.5 ml) for 60 min. at 37 °C. Catalase is added to remove residual H_2O_2 , then ascorbic acid (0.5 ml of 0.02%) was added and mixed for 5 min. Trichloroacetic acid (0.5 ml of 20%) was then added to precipitate proteins. The 1.5 ml mixture was centrifuged, and the supernatant (1 ml) was added to ammonium acetate buffer (0.45 ml) and ferrozine reagent (50 μl). The iron colour complex was measured at 560 nm. The amount of iron released was calculated by measuring the absorbance of an iron standard (500 μg/dl) (0.5 ml), treated as described above, against blank (0.5 ml H_2O). ($A_{unknown}/A_{standard}$ * 500).

2.5 Results

Preparation of PolyHb and PolyHb-SOD-Catalase

To determine the degree of polymerization, samples were analyzed by gel filtration chromatography. The molecular weight distributions were the same for PolyHb and PolyHb-SOD-CAT (Fig. 2.1). About 70% of the molecules were eluted in the molecular weight range greater than 600 kD, ~ 15% in the region between 600 kD - 66 kD, and the remaining are eluted around 66 kD. The ratio of hemoglobin to SOD and catalase (as mg/ml) was 1 : 0.009 : 0.0045. The added SOD and catalase, therefore, is not expected to significantly change the molecular weight distribution after being crosslinked with hemoglobin. Detailed analysis of the polymerized product is in progress.

Scavenging of superoxide

Experiments were designed to find out whether PolyHb-SOD-CAT eliminates enzymatically-generated superoxide. Results are shown in Fig. 2.2A. The presence of SOD activity was indicated by an inhibited rate of cytochrome c reduction. The initial rate of cytochrome c reduction was 0.56 ± 0.08 nmoles cyt. c/min for PolyHb-SOD-CAT compared to 2.13 ± 0.26 nmoles cyt. c/min for PolyHb. Interference due to the presence of hemoglobin components in the reaction mixture was ruled out since the rate of reduction in PolyHb mixtures was similar to the reduction rate in buffer alone. It was also found that the superoxide scavenging activity of the polymerized solution varies

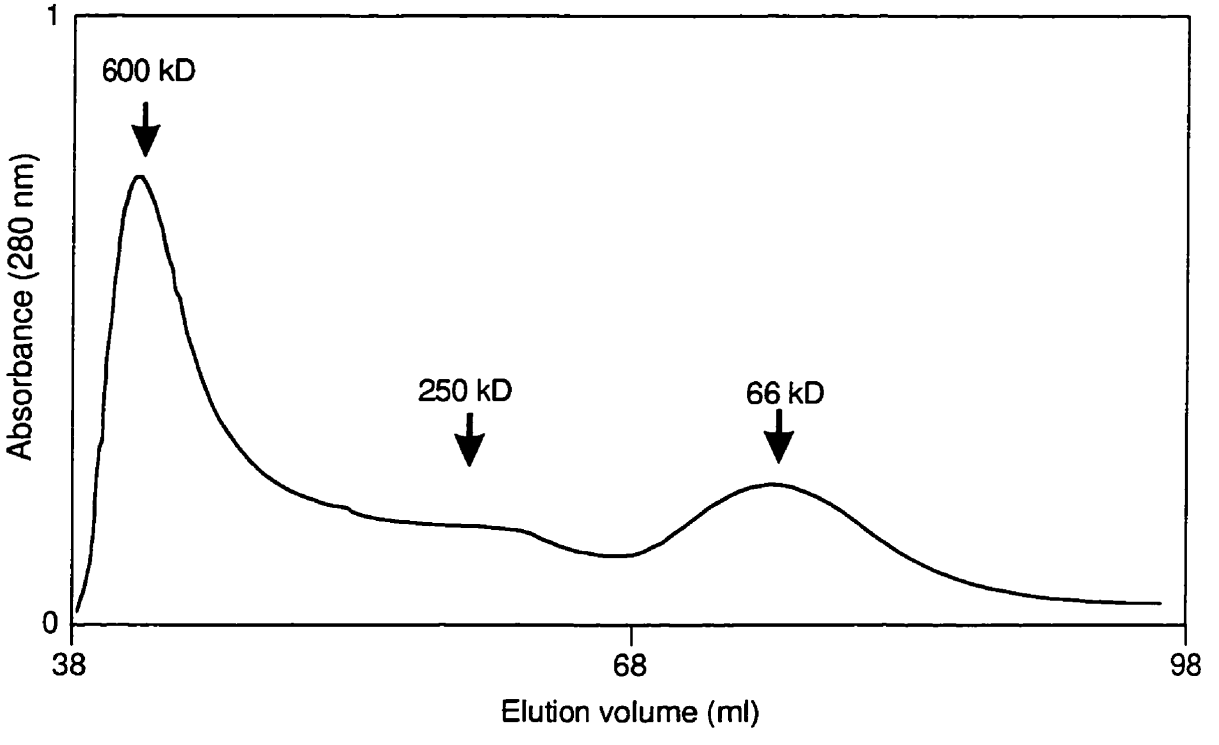

Figure 2.1 Typical elution profile of PolyHb or PolyHb-SOD-CAT (1 ml sample) run on a Sephadex G-200 1.6 cm x 70 cm column, V_T = 97.5 ml, equilibrated with 0.1 M Tris-HCl, pH 7.5, and eluted at 12 ml/hr.

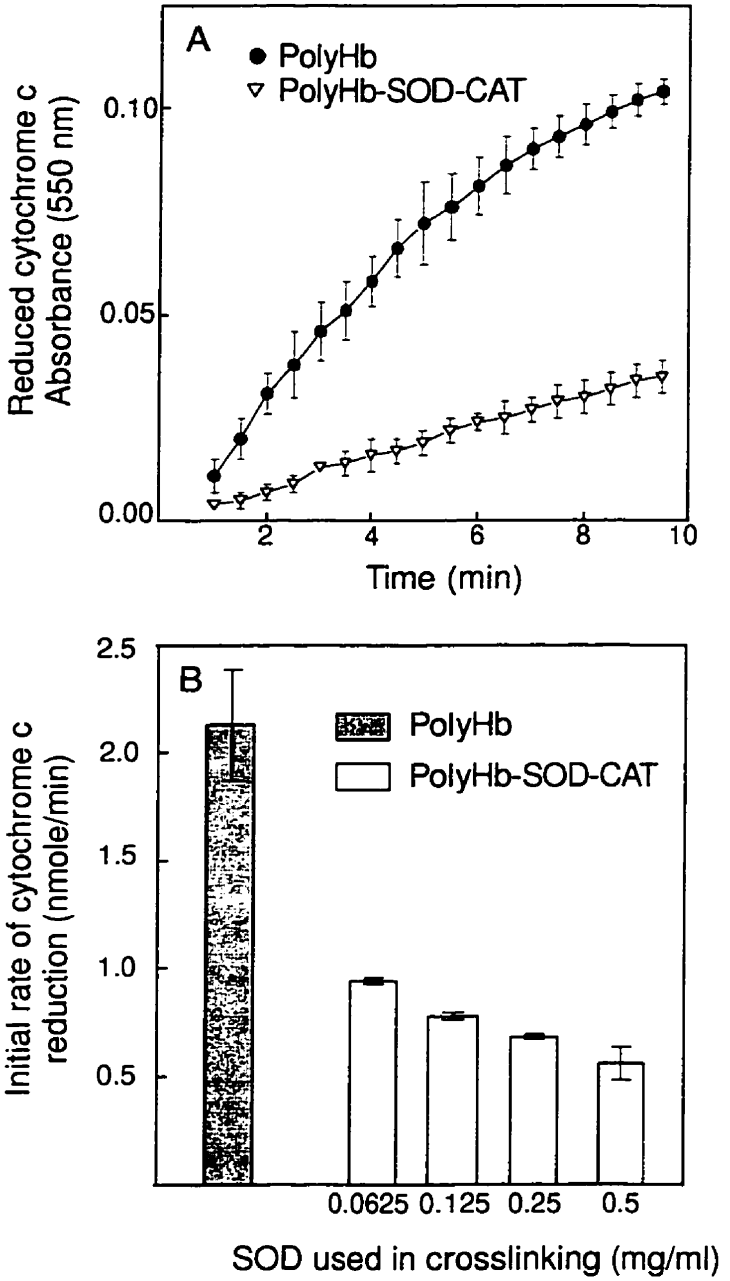

Figure 2.2 (A) Reduction of cytochrome c, by xanthine/xanthine oxidase-generated superoxide, in the presence of PolyHb or PolyHb-SOD-CAT (5 μM). 3 ml reaction volumes with 50 mM potassium phosphate containing 0.1 mM EDTA at pH 7.8. The reaction was started by adding xanthine oxidase. (B) The initial rate of cytochrome c reduction in reaction volumes containing either PolyHb (5 μM) or PolyHb-SOD-CAT (5 μM) prepared with different concentrations of superoxide dismutase. Error bars represent standard error of triplicate measurements.

with the SOD concentration, used during crosslinking (Fig. 2.2B).

Scavenging of hydrogen peroxide

Experiments were also done to determine whether PolyHb-SOD-CAT eliminates reagent hydrogen peroxide. Results are shown in Fig. 2.3A. In reaction mixtures containing PolyHb-SOD-CAT, only about 20% of the added H_2O_2 was recoverable at each concentration studied, whereas more than 95% was detectable in PolyHb mixtures. With increasing H_2O_2 concentrations, reactions between PolyHb itself and H_2O_2 became more evident (Fig. 2.3A). It was also observed that the H_2O_2 scavenging activity of PolyHb-SOD-CAT varies with the catalase concentration used during crosslinking (Fig 2.3B).

Monitoring absorbance spectra following oxidative challenge

Absorbance spectral recordings were used to monitor the reactions of oxygen free radicals with the hemoglobin components of the crosslinked solutions. Results obtained from reactions with H_2O_2 are shown in Fig. 2.4 A-D. Following incubation with equimolar H_2O_2 (10 μM), the spectral changes of PolyHb reflect the oxidation of ferrous (Fe^{2+})-heme producing ferric (Fe^{3+})-heme. With excess H_2O_2, the heme moieties of PolyHb were rapidly degraded. The absorbance spectra of PolyHb-SOD-CAT were minimally affected, indicating these reactions are minimized due to the elimination of H_2O_2. Similar results were recorded following oxidative challenge with exogenous $O_2^{\bullet-}$ via xanthine/xanthine oxidase (data not shown). It was observed, from the spectra

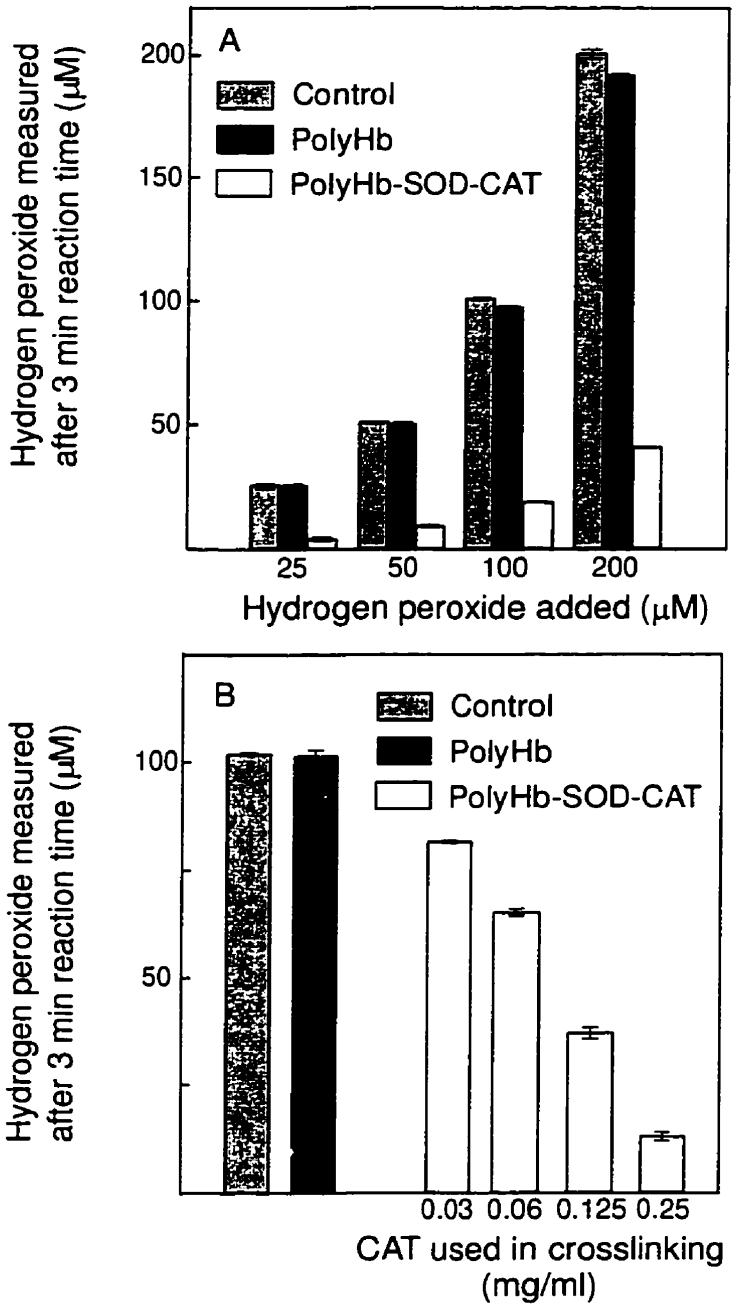

Figure 2.3 (A) Peroxidase-catalyzed measurement of H_2O_2 in 3 ml reaction volumes containing added H_2O_2 and either PolyHb (5 μM) or PolyHb-SOD-CAT (5 μM). (B) Hydrogen peroxide measured, 3 min after the addition of 100 μM H_2O_2, in either PolyHb (5 μM) or PolyHb-SOD-CAT (5 μM) prepared with different catalase concentrations. Error bars represent standard error of triplicate measurements.

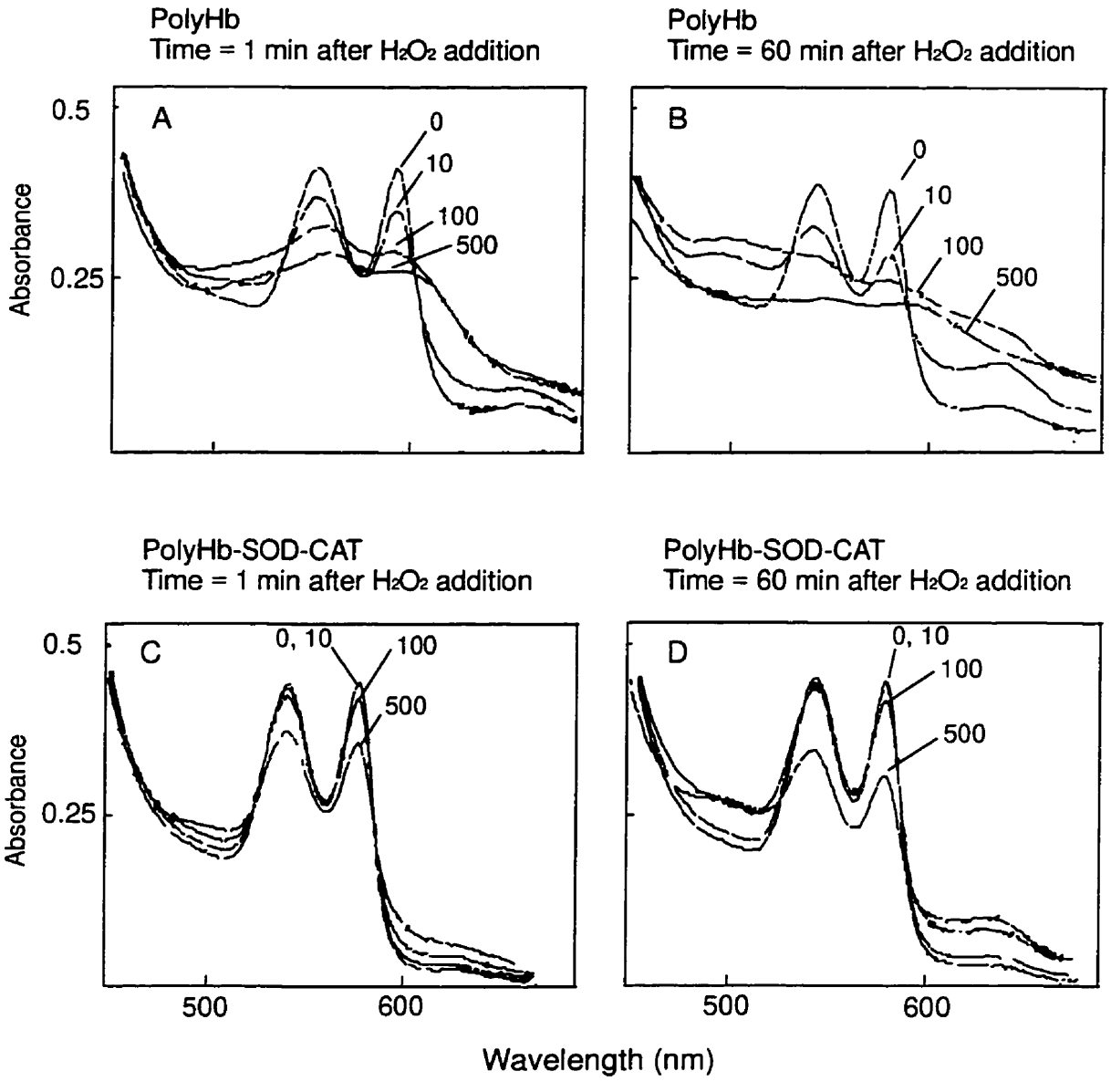

Figure 2.4 Absorbance spectra of PolyHb and PolyHb-SOD-CAT (10 μM) following H_2O_2 addition of 0, 10, 100, and 500 μM.

recorded in the absence of H_2O_2, that the PolyHb solution contained higher starting levels of methemoglobin compared to PolyHb-SOD-CAT. This shows that SOD-catalase may also provide oxidative protection during the preparation and/or storage of modified hemoglobin solutions.

Iron measurement

Experiments to quantify the release of iron from PolyHb or PolyHb-SOD-CAT, due to H_2O_2 challenge, were also performed. Results are shown in Fig. 2.5A. With H_2O_2 additions of 10 to 500 μM, it is estimated that from 2 to 37% of the total iron in PolyHb (15 μM) was "freed" and made detectable by ferrozine assay. Over the same H_2O_2 concentrations, less than 1% was released from PolyHb-SOD-CAT. It was also found that the catalase concentration used during crosslinking directly affects the amount of iron released during H_2O_2 incubation (Fig. 2.5B).

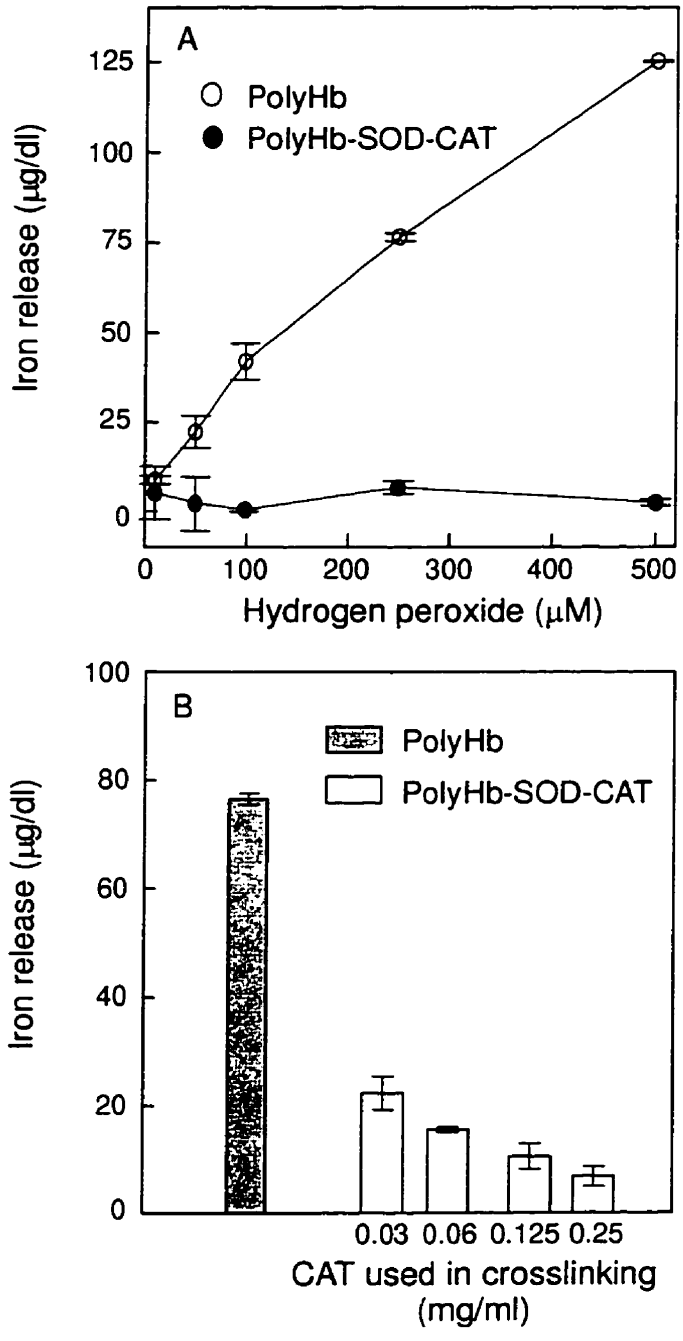

Figure 2.5 (A) The release of iron, measured by ferrozine assay, from PolyHb (15 µM) or PolyHb-SOD-CAT (15 µM) incubated with H_2O_2 for 1hr. at 37 °C. (B) Iron release from either PolyHb (15 µM) or PolyHb-SOD-CAT (15 µM) prepared with different catalase concentrations, following incubation with 250 µM H_2O_2. Error bars represent standard error of triplicate measurements.

2.6 Discussion

Modified hemoglobin solutions, with effective oxygen-delivering characteristics, have potential applications in several clinical and/or experimental settings. However, certain toxicity issues still need to be resolved. Hemoglobin-based solutions may aggravate oxidative stress in biological systems. The two main routes by which modified hemoglobin solutions may promote oxidative stress include; a) an increase in oxygen free radicals, and b) providing catalytic heme or iron, driving the production of highly reactive hydroxyl or ferryl radicals.

Under normal circumstances, SOD and catalase in the red blood cell help prevent oxidative degradation of hemoglobin and protect other red cell components. Modified hemoglobin with cross-linked SOD-catalase may imitate the conditions in the red blood cell, and thus reduce hemoglobin-related toxicity. This may be important because in the circulation, hemoglobin-based solutions, devoid of antioxidant defense mechanisms, are likely to undergo oxidative attack and participate in the formation of harmful secondary free radicals via Fenton chemistry [5]. Some suggest that hemoglobin itself can act as a Fenton catalyst [17], while others disagree [18]. The idea that free hemoglobin may be harmful is further supported by the existence of protective plasma proteins (e.g. haptoglobin, hemopexin, and ceruloplasmin) that eliminate or reduce the reactivity of hemoglobin and/or its breakdown products [17,19].

There are several reasons we chose to cross-link both SOD and catalase, instead of one or the other. The abnormal production of superoxide may be a significant

mechanism in hemoglobin-induced oxidative stress. Superoxide dismutase eliminates superoxide, but in the process produces hydrogen peroxide. The use of catalase avoids the accumulation of hydrogen peroxide. Others suggest that the "overscavenging" of superoxide may be partly responsible for the observed ineffectiveness of antioxidant treatments based solely on superoxide dismutase [10]. Crosslinking the enzymes to PolyHb is necessary since free SOD and catalase are rapidly removed from the circulation (10 and 20 min., respectively) [9]. Furthermore, unless cross-linked to PolyHb, these enzymes do not locate at sufficient proximity to PolyHb, and thus are less likely to provide adequate protection. This may explain why adding free SOD and catalase to free hemoglobin failed to show beneficial effects [20].

Nitric oxide produced by endothelial cells, diffuses into smooth muscle cells, and activates processes leading to vasodilation. Some modified hemoglobin solutions are known to exert a vasoconstrictive effect, generally attributed to the direct binding of nitric oxide (NO) to the heme portion of hemoglobin [21]. However, it has also been shown that oxygen free radicals can inactivate NO, and cause vasoconstriction [22]. Reports show that free radical scavengers reduce blood pressure (MAP) in situations of oxidative stress, presumably by preventing free radical inactivation of NO [22,23]. Since modified hemoglobins promote the production of free radicals, this may contribute to its vasoconstrictive effect. Another possibility is that heme released from broken down hemoglobin can bind nitric oxide. Heme is a hydrophobic molecule which can insert itself in the membranes of endothelial cells [24]. Crosslinked SOD-catalase,

therefore, may actually reduce vasoconstriction by scavenging free radicals and/or by blocking the breakdown of hemoglobin, thus avoiding the inactivation of nitric oxide.

2.7 References

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2.8 Appendix: Hemoglobin measurement

Drabkin's method was used to measure hemoglobin concentration[1]. Hemoglobin and its derivatives, except sulfhemoglobin, are oxidized to methemoglobin by ferricyanide. Methemoglobin is then converted to cyanomethemoglobin by its reaction with cyanide (Drabkin's reagent). The red coloured complex that is produced has a peak absorbance at 540 nm, and is proportional to hemoglobin concentration. Drabkin's reagent contains sodium bicarbonate (1 g/l), potassium cyanide (0.052 g/l) and potassium ferricyanide (0.13 g/l). Cyanomethemoglobin standard (0.08 g/dl) is supplied in kit. Standard and reagent solution are combined to give working standards with hemoglobin equivalency (g/dl) based on dilution sample factor of 1:251. The procedure is performed as follows. Zero the spectrophotometer with 1 ml cuvettes containing reagent solution. Add 20 µl of sample to 5 ml reagent solution. After mixing, wait 3 min, transfer to cuvettes, then measure absorbance at 540 nm. Determine concentration of sample from standard curve.

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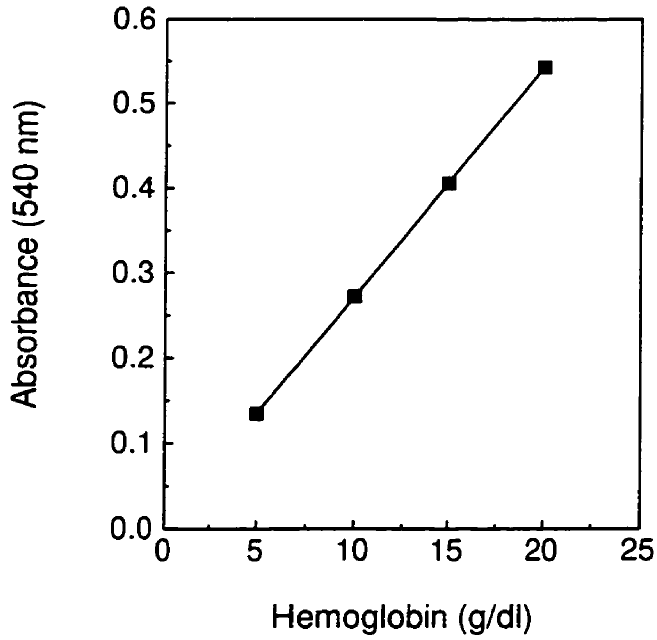

Figure 2.6 Standard curve for hemoglobin determination

2.9 Appendix: Horseradish peroxidase-based H_2O_2 assay

The principal of this assay is that hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol, in the presence of horseradish peroxidase, to produce a quinoneimine dye with an absorbance peak at 505 nm[1]. The reagent solution (100 ml) contained 0.234 g of phenol (or 0.26 ml of 90% liquid phenol), 0.1g of 4-aminoantipyrine, 1 ml of 0.1 M phosphate buffer pH 6.9, 20 nM of horseradish peroxidase (80 µl of 1 mg/ml phosphate buffer), and 2.5 µ M of H_2O_2. Small quantity of H_2O_2 prevents calibration curve from having a negative y-intercept due to 4-aminoantipyrine impurities[1]. Reaction volumes were prepared containing 5 µM (0.032 g/dl or ~ 20 µM as heme) of PolyHb or PolyHb-SOD-CAT (1.5 ml) and 0.3 ml of hydrogen peroxide (giving desired concentration). After allowing mixture to stand for 3 min at 22 °C, 1.2 ml of the reagent solution is added. After 3-4 min at 22 °C, the absorbance is measured at 505 nm.

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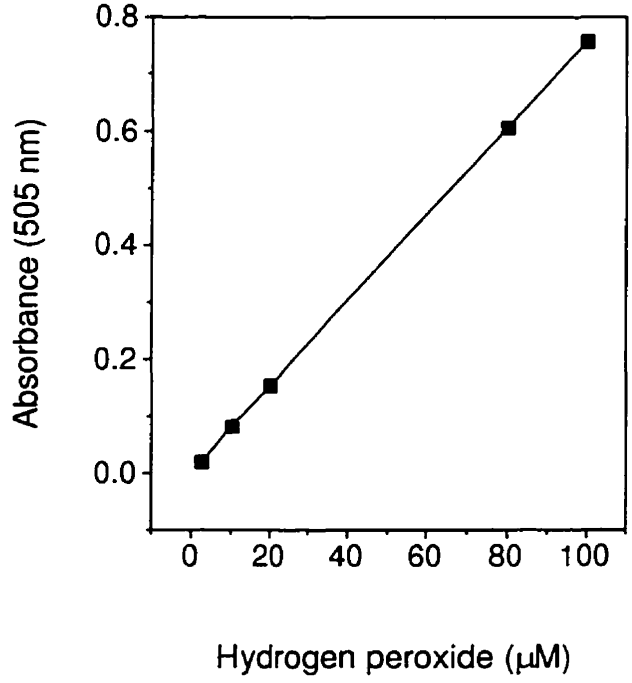

Figure 2.7 Standard curve for hydrogen peroxide assay based on horseradish peroxidase

2.10 Appendix: Sephadex G-200: calibration curve

The molecular weight exclusion limits of the Sephadex G-200 column were 5 - 600,000 Daltons. The void volume (V_O) of the Sephadex G-200 column was found to be 40 ml determined by the initial elution peak of a sample of Blue Dextran 2000. Kav values were calculated from the equation, V_E-V_O / V_T - V_O. Where V_T represents effective bed volume (radius$^2\pi$ x gel height), V_E represents elution volume of sample. Calibration curve was plotted as Log molecular weight versus Kav values. The calibration proteins were thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), albumin (66 kD), carbonic anhydrase (29 kD), cytochrome c (12 kD).

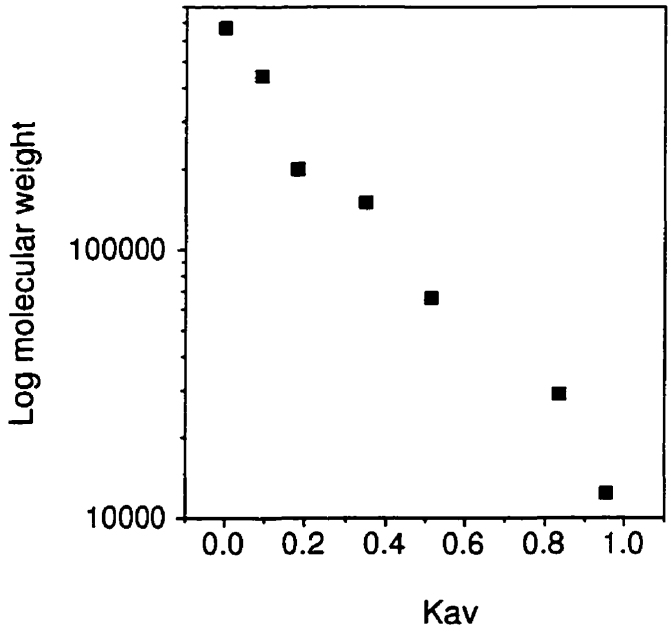

Figure 2.8 Protein calibration curve for Sephadex G-200 column.

2.11 Appendix: Iron measurement by ferrozine method

Ferrozine, a sulfonated derivative of diphenyltriazine (3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine) forms a water-soluble magenta complex with ferrous iron (Fe^{2+}) with an absorption maximum at 560 nm. Ferrozine (0.85 % w/v) and the iron standard (500 g/dl), both in hydroxylamine hydrochloride solution, were obtained from Sigma Chemical Co, St. Louis, MO. The reducing agent ascorbic acid used in this assay converts ferric iron to the detectable ferrous form. In this assay, I used trichloroacetic acid to precipitate hemoglobin prior to reaction with ferrozine. Ferric iron is co-precipitated by trichloroacetic acid whereas ferrous iron is not[1]. Therefore, ascorbic acid is added 5 min prior to precipitation step. The amount of iron in samples was calculated by measuring the absorbance of the iron standard (500 μg/dl) (0.5 ml) against treated blank (0.5 ml H_2O), and then using the following equation; A560unknown * A560standard^{-1} * 500. There was no difference in absorbance for control PolyHb and PolyHb-SOD-CAT samples, both ranged from 0.06 - 0.063 when zeroed against untreated water. Whereas a water sample taken through precipitation procedure gave background absorbance of 0.057, this value was subtracted from sample readings.

Carter P. *Analytical Biochemistry* 40:450-458, 1971.

CHAPTER 3

Structural and Functional Aspects of a Novel Red Blood Cell Substitute Based on Crosslinked Hemoglobin-Superoxide Dismutase-Catalase

3.1 Link to previous chapter 2

The purpose of this chapter is to further define the structural and functional properties of PolyHb-SOD-CAT. In the previous chapter, I show that superoxide dismutase and catalase retain their free radical scavenging ability. In this chapter, I study in more detail the effect of glutaraldehyde on SOD and catalase activity. In the previous chapter, I analyzed PolyHb-SOD-CAT containing varying amounts of each enzyme, however in those experiments the ratio of SOD : catalase was constant. In this chapter, I study the oxidation of hemoglobin incubated with varying ratios of SOD : catalase. This data provides information about the ratios that are effective in preventing hemoglobin oxidation. In the previous chapter, I showed molecular size distribution of modified hemoglobin using Sephadex column gel filtration method. In this chapter, I use a more efficient and accurate technique of high performance gel filtration. A central hypothesis in this thesis is that intermolecular polymerization increases the circulation time of superoxide dismutase and catalase. To test this hypothesis, I perform circulation time studies with PolyHb-SOD-CAT in rats.

3.2 Abstract

This study describes the structural and functional properties of a novel red blood cell substitute based on crosslinked hemoglobin, superoxide dismutase, and catalase (PolyHb-SOD-CAT). Studies show that glutaraldehyde polymerization with 8:1 and 16:1 glutaraldehyde/hemoglobin ratio results in minimal losses in final superoxide dismutase and catalase activity. We also evaluate the effect of varying SOD/CAT activity ratios on the susceptibility of hemoglobin to oxidation in the presence of superoxide (xanthine-xanthine oxidase) and hydrogen peroxide (glucose-glucose oxidase). We analyze the circulation half-life of hemoglobin, SOD and catalase in Sprague-Dawley rats. Our results show that the degree of polymerization correlates with the circulation time of all three proteins. High performance liquid gel filtration chromatography is used to analyze molecular size distribution of polymerized products. These studies demonstrate a hemoglobin-based red blood cell substitute with antioxidant properties. Given current toxicity questions, these antioxidant capabilities may improve the suitability of modified hemoglobin in vivo.

3.3 Introduction

Potential red blood cell substitutes based on chemically and genetically modified hemoglobin solutions are currently undergoing clinical evaluation [1-4]. Early problems associated with oxygenation properties, circulatory half-life, and purity have been resolved [1-4]. For certain applications, the pro-oxidant activity of modified hemoglobin have raised several concerns [5,6].

Hemoglobin can readily generate or interact with free radicals [6-11], and promote lipid peroxidation processes [12-14]. Several lines of evidence suggest this pro-oxidant nature mediates the toxicity of free and modified hemoglobin in vitro [15-17] and in vivo [18-22]. Significant concern focuses on the possibility that modified hemoglobin exacerbate oxidative stress associated with ischemia-reperfusion injury [5,17,20,23,24].

Autoxidation of hemoglobin to methemoglobin is a constant source of superoxide radical ($O_2^{\cdot-}$) in erythrocytes [8]. Superoxide can spontaneously or enzymatically, via superoxide dismutase, be converted to hydrogen peroxide (H_2O_2). Superoxide dismutase, catalase, and glutathione peroxidase normally ensure the detoxification of reactive oxygen species in the red blood cell. Due to toxicity that may arise from endotoxin and other potential contaminants, highly purified hemoglobin is used in the preparation of modified hemoglobin blood substitutes [2,3,25,26]. Therefore, acellular hemoglobin-based oxygen carriers lack important antioxidant defense systems. In the circulation, hemoglobin molecules may be highly susceptible to interactions with endogenous oxidants especially during activation of phagocytes or during

reoxygenation following ischemia [27-29]. Recently, we proposed a strategy that combines hemoglobin and the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) [30,31].

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (SOD)}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (CAT)}$$

This approach may limit the pro-oxidant effects of hemoglobin by detoxifying reactive oxygen species produced by hemoglobin, and by preventing reactions with other endogenously produced oxidants. Native superoxide dismutase and catalase have short half-lives in circulating blood. Therefore, the simple addition of these enzymes to modified hemoglobin may not produce the desired protective benefits. Our initial efforts have centered on using glutaraldehyde to non-specifically polymerize solutions containing hemoglobin, SOD, and catalase [30,31]. As reported here, this scheme extends the circulation half-lives of hemoglobin, SOD, and catalase. Furthermore, this approach may increase the efficiency of protection by maintaining a close association between antioxidants and hemoglobin, similar to the normal situation in the red blood cell. Previously, we show that SOD and catalase retain their ability to scavenge superoxide and hydrogen peroxide following polymerization with hemoglobin [30]. We also show that SOD and catalase significantly protects modified hemoglobin from autoxidation and oxidative damage [30-32]. Recently, we report that reperfusion with

PolyHb-SOD-CAT reduces hydroxyl radical generation compared to PolyHb in rat models of isolated perfused hindlimb [23] and intestine [24].

In the present study, we further evaluate the structural and functional properties of PolyHb-SOD-CAT. We study in more detail the effect of glutaraldehyde polymerization on enzymatic activities of superoxide dismutase and catalase. We also assessed the effects of varying SOD-CAT ratios in relation to the oxidation of hemoglobin. The degree of polymerization is analyzed by high performance gel filtration, and indirectly by determining the circulation half-lives of SOD, catalase, and hemoglobin following infusion of PolyHb-SOD-CAT in rats.

3.4 Materials and methods

Materials

Purified bovine hemoglobin was purchased from Biopure Corporation, Boston, MA. Bovine erythrocyte superoxide dismutase (3300 units/mg manufacturer's stated activity), determined by cytochrome c reduction assay, and bovine liver catalase (72575 units/ml manufacturer's stated activity), determined by hydrogen peroxide decomposition at 240nm, were obtained from ICN Biomedicals, Aurora, OH. Cytochrome c from horse heart (type III), bovine milk xanthine oxidase, xanthine, and glucose oxidase from *Aspergillus niger* were also purchased from ICN Biomedicals. Glutaraldehyde (25%) was obtained from Polyscienes, Warrington, PA. Hemoglobin assay kit was purchased from Stanbio Laboratory, San Antonio, TX. Other chemicals of reagent and HPLC grade were obtained from BDH, Toronto, ON.

Preparation of PolyHb and PolyHb-SOD-CAT

Reaction mixtures were prepared containing hemoglobin (7g/dl), SOD (1050 U/ml), and catalase (21,000 U/ml) in 50mM sodium phosphate, pH 7.4. The final ratio was 1g Hb : 15,000 U SOD : 300,000 U CAT. In PolyHb mixtures, an equivalent volume of buffer replaced enzyme addition. Prior to the start of crosslinking, lysine was added at a molar ratio of 10:1 lysine/hemoglobin. Crosslinking reaction was started with the addition of glutaraldehyde at molar ratio of 8:1 or 16:1 glutaraldehyde/hemoglobin. Glutaraldehyde was added in four equal aliquots over a

period of 10-15 min. Non-crosslinked preparations consisted of the identical mixtures except buffer was added instead of glutaraldehyde. After 3.5 hr at 4 C° under aerobic conditions with constant stirring, reaction was stopped with excess lysine at a molar ratio of 100:1 lysine/hemoglobin. Additional reduction step with sodium borohydride was not performed in order to minimize methemoglobin formation. Solutions were dialyzed in physiological saline solution and passed through sterile 0.2 μM filter. Aliquots (300 μl) of the 8:1 crosslinked preparation were concentrated using 100 kD microconcentrators (Amicon, Beverly, MA). Samples were centrifuged at 3000 g for 25 min at 23 °C. After first spin, retentate was collected, diluted, and spun again. Final retentates were diluted and pooled. Hemoglobin concentration was determined by cyanomethemoglobin measurement at 540nm.

HPLC gel filtration analysis of molecular size distribution

Samples (10 μl of 1 mg/ml) were analyzed by high performance gel filtration chromatography. The HPLC setup consisted of an automatic injector (Waters Wisp 712B), solvent delivery system (Waters 6006A), dual-channel integrator (Waters Data Module 746), and UV/VIS detector (Waters 450). Molecular weight gel filtration analysis was conducted using a BioSep Sec S-3000 column (Phenomenex, Torrance, CA). The mobile phase consisted of 0.01 M potassium phosphate, 0.15M ammonium acetate, pH 7.0. The flow rate was 1 ml/min. The detector wavelength was set at 280 nm.

Circulation time studies

Fasted male Sprague-Dawley rats (250-275 g, Charles River, St-Constant, QC) were anesthetized 65 mg/kg sodium pentobarbital (Somnotol, Becton Dickinson, NJ). Femoral artery and vein were cannulated with PE-10 and PE-50 polyethylene tubing (Clay Adams, Dickinson, NJ) respectively. Blood pressure and heart rate were recorded via femoral artery connected to a Statham P23AA pressure transducer and Grass 79D polygraph recorder (Grass Instrument Co., Quincy, MA). Heparinzed saline (150 units/ml) was administered via the femoral vein prior to start of the experiment. After 15 min stabilization period, test solution (300 mg hemoglobin/kg rat) was infused via femoral vein. Femoral venous samples (0.5 ml) were withdrawn at indicated time intervals. Samples were centrifuged at 15,600 g for 5 min. Recovered plasma was stored at -80 °C.

Superoxide dismutase activity

Superoxide dismutase activity was determined by cyctochrome c reduction assay [33] with slight modifications. One unit of SOD is defined as the amount of enzyme that inhibits the rate of cytochrome c reduction by 50% under specified conditions. The reagent solution consisted of xanthine (50 μM), cytochrome c (10 μM), and catalase (500 units/ml) in 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.8. Catalase added to reagent solution prevented interference due to the hemoglobin-hydrogen peroxide interactions. Reaction volumes were prepared containing test sample (25 μl of

0.6 g/dl), reagent solution (1.45 ml), and xanthine oxidase (25 µl of 0.2 units/ml) to start the reaction. The initial rate of cytochrome c reduction was monitored at 550 nm with a Perkin-Elmer Lambda 4B Spectrophotometer. Standard curve for SOD was prepared in the presence of hemoglobin (0.1 mg/ml). SOD activity in polymerized preparations was compared to non-crosslinked samples. For activity determinations in circulation time experiments, 25 µl of plasma was assayed directly.

Catalase activity

Catalase activity was assessed by measuring the decomposition of hydrogen peroxide at 240 nm [34]. Spectrophotometer was zeroed with two cuvettes (1 ml) containing test sample (10 µl of 0.6 g/dl) in 50mM potassium phosphate buffer, pH 7.0. In experimental samples, hydrogen peroxide (250 µl of 30 mM) was added instead of buffer, and the absorbance at 240 nm was monitored for 36 sec. The disappearance rate of hydrogen peroxide was used as a measure of catalase activity. For activity determinations in circulation time experiments, 10 µl of plasma was assayed directly.

Ratio of SOD/CAT and hemoglobin oxidation

Reaction volumes were prepared containing 50 µM (as heme) in 50 mM potassium phosphate, pH 7.4 and varying ratios of SOD/CAT (0, 0.01, 100) with final enzyme activities of 3 or 300 units. Samples also contained either xanthine (100 µM)/xanthine oxidase (10 mU/ml) or glucose (10 mM)/ glucose oxidase (10 µg/ml).

Reactions (total volume 1 ml) were started with the addition of the oxidase enzymes at 23 °C. Absorbance spectra (500-700 nm) were recorded at 1 scan/min for 25 min using a Perkin Elmer Lambda B spectrophotometer.

3.5 Results

Effect of polymerization on SOD and catalase activity

Figure 3.1 shows activity measurements of SOD and catalase. The effect of glutaraldehyde polymerization on the final activity of SOD and CAT is summarized in Figure 3.2. Polymerization with 8:1 glut/Hb resulted in minimal losses of activity. Increasing the polymerization did not produce significant losses of SOD or catalase activity. Analysis of oxygen dissociation curves shows these enzymes did not alter oxygenation characteristics of modified hemoglobin.

Ratio of SOD-CAT and oxidative hemoglobin reactions

Figure 3.3 represents hemoglobin absorbance spectra collected during oxidative challenge of hemoglobin and varying ratios of SOD and catalase. Spectra in Fig. 3.3A-C are obtained during a flux of superoxide catalyzed by xanthine-xanthine oxidase. In Fig. 3.3A, hemoglobin (Fe^{2+}) oxidation is shown by gradual decrease in absorbance at 540 and 577 nm, and methemoglobin (Fe^{3+}) appears as absorbance peak at 630 nm. In Fig. 3.3B, the SOD/CAT ratio of 0.01 prevents this oxidation, while the SOD/CAT ratio of 100 produces no protective effect (Fig. 3.3C). Fig. 3.3D-F are obtained during a flux of hydrogen peroxide generated by glucose-glucose oxidase. In Fig. 3.3D, hydrogen peroxide produces the ferryl (Fe^{4+}) intermediate indicated by the appearance of new peaks at 545 and 580 nm [11]. The SOD/CAT ratio of 0.01 reduces this oxidation, while a ratio of 100 produces no protective effect.

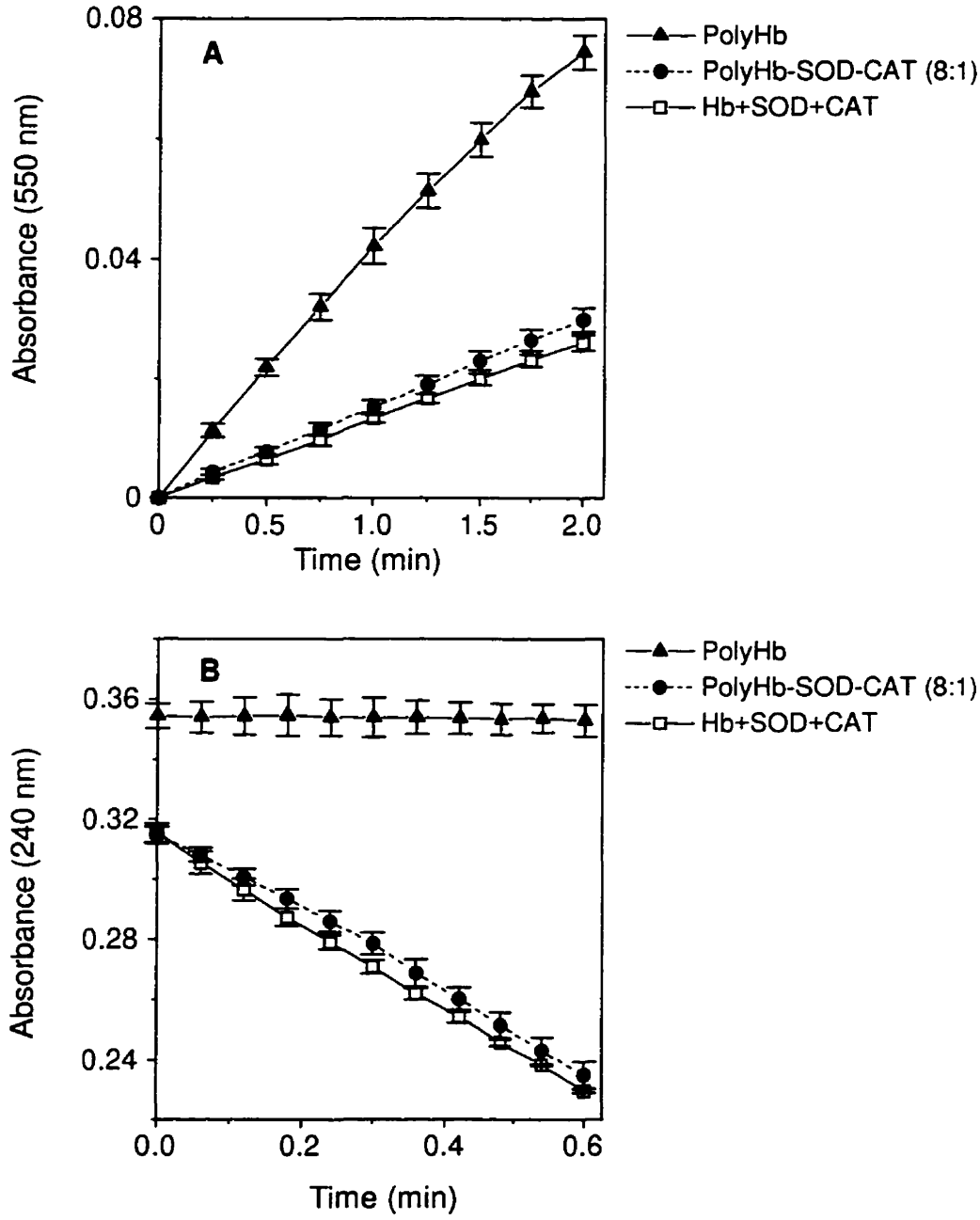

Figure 3.1 (A) SOD activity measurements. An inhibited rate of cytochrome c reduction is a measure of SOD activity. Curves represent the mean of five determinations for each sample. (B) Catalase activity measurements. Rate of hydrogen peroxide disappearance at 240 nm is a measure of catalase activity. Curves represent the mean of three determinations for each sample.

Sample	% SOD Activity retained	% CAT Activity retained
Hemoglobin + SOD + Catalase	100	100
PolyHb-SOD-CAT 8:1 Glut:Hb	90	99
PolyHb-SOD-CAT 16:1 Glut:Hb	85	95

Figure 3.2 Superoxide dismutase and catalase activity retained following polymerization reaction based on 100% values of non-crosslinked preparations.

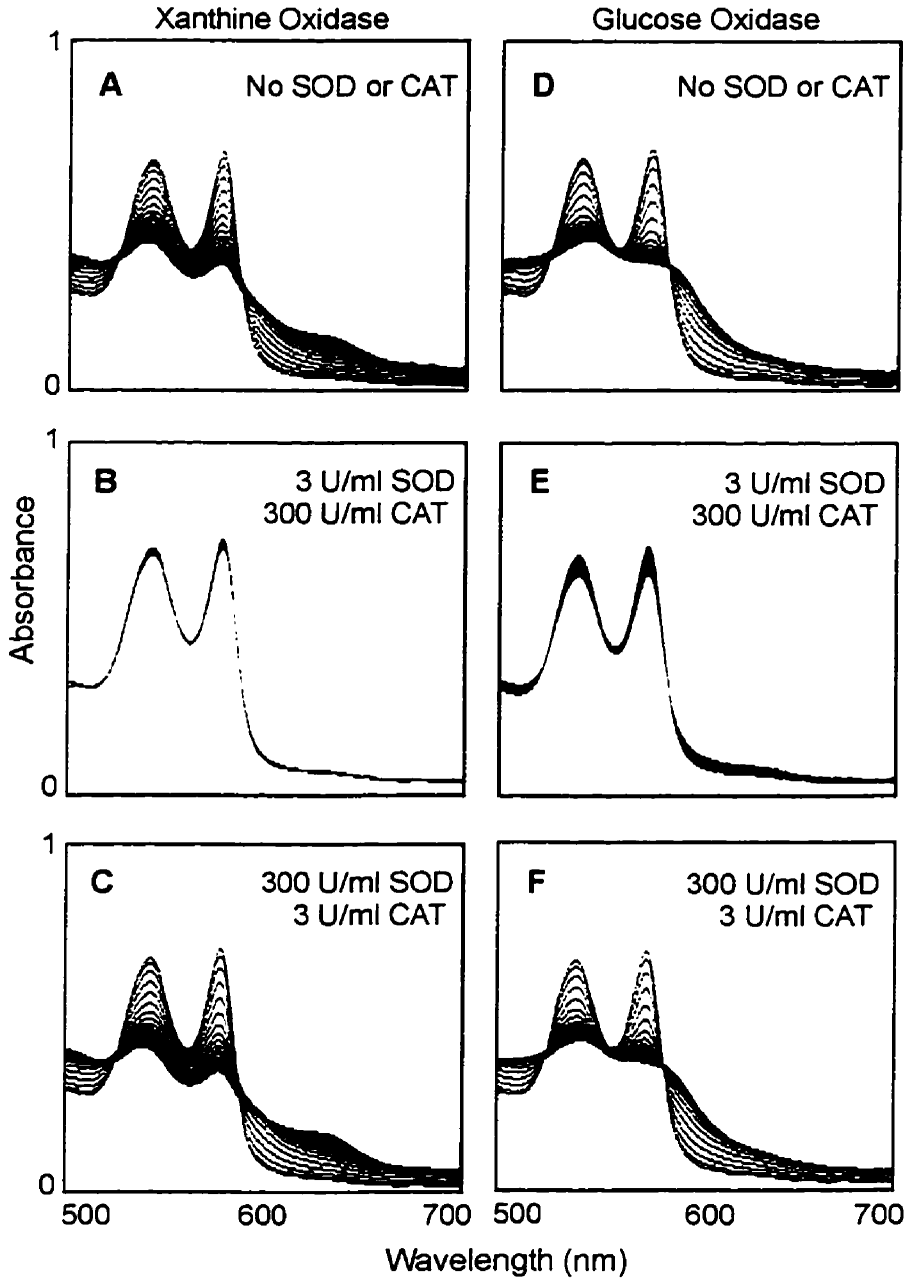

Figure 3.3 Representative absorbance spectra of reaction mixtures containing 50 μM of Hb and 100 μM of xanthine, and 10 mU/ml of xanthine oxidase (A-C) or 10 mM of glucose and 10 μg/ml glucose oxidase (D-F). SOD/CAT activities included in each individual mixture are shown. Each panel represents a series of spectra collected over a period of 20 min at 1 scan/min.

Molecular size distribution by HPLC gel filtration

Figure 3.4 shows the molecular size elution profiles of modified hemoglobin. Analysis of the hemoglobin, SOD, and CAT mixture reveals one peak eluting at about 19.6 min that indicates the tetramer hemoglobin fraction. At the present concentrations, SOD and CAT are not detectable by this method. In the 8:1 glut/Hb preparation, three peaks are discernible at 19.6, 17.6, and 16.4 min. Ultrafiltration of the 8:1 preparation through 100 kD cutoff membranes is primarily aimed at removing SOD (32 kD). This two-pass filtration was not designed to eliminate tetrameric hemoglobin (~ 65 kD), although minor decrease in tetramer hemoglobin peak and slight widening of the 16.4 min peak is noted. Preparation with 16:1 glutaraldehyde/hemoglobin produces higher degree of polymerization. Elution profiles show five peaks eluting at 19.6, 18.6, 16.4, 16.2, and 11.2 min. The 11.2 min peak is the void volume, and indicates polymers with molecular size >700,000 kD.

Circulation time analysis

Figures 3.5, 3.6 and 3.7 shows the circulation time of superoxide dismutase, catalase, and hemoglobin in Sprague Dawley rats following the injection of:

(1) native hemoglobin, SOD, and catalase

(2) PolyHb-SOD-CAT, prepared with 8:1 glutaraldehyde/hemoglobin

(3) PolyHb-SOD-CAT, prepared with 16:1 glutaraldehyde/hemoglobin

(4) PolyHb-SOD-CAT (8:1 preparation), ultrafiltered through 100 kD cutoff membranes.

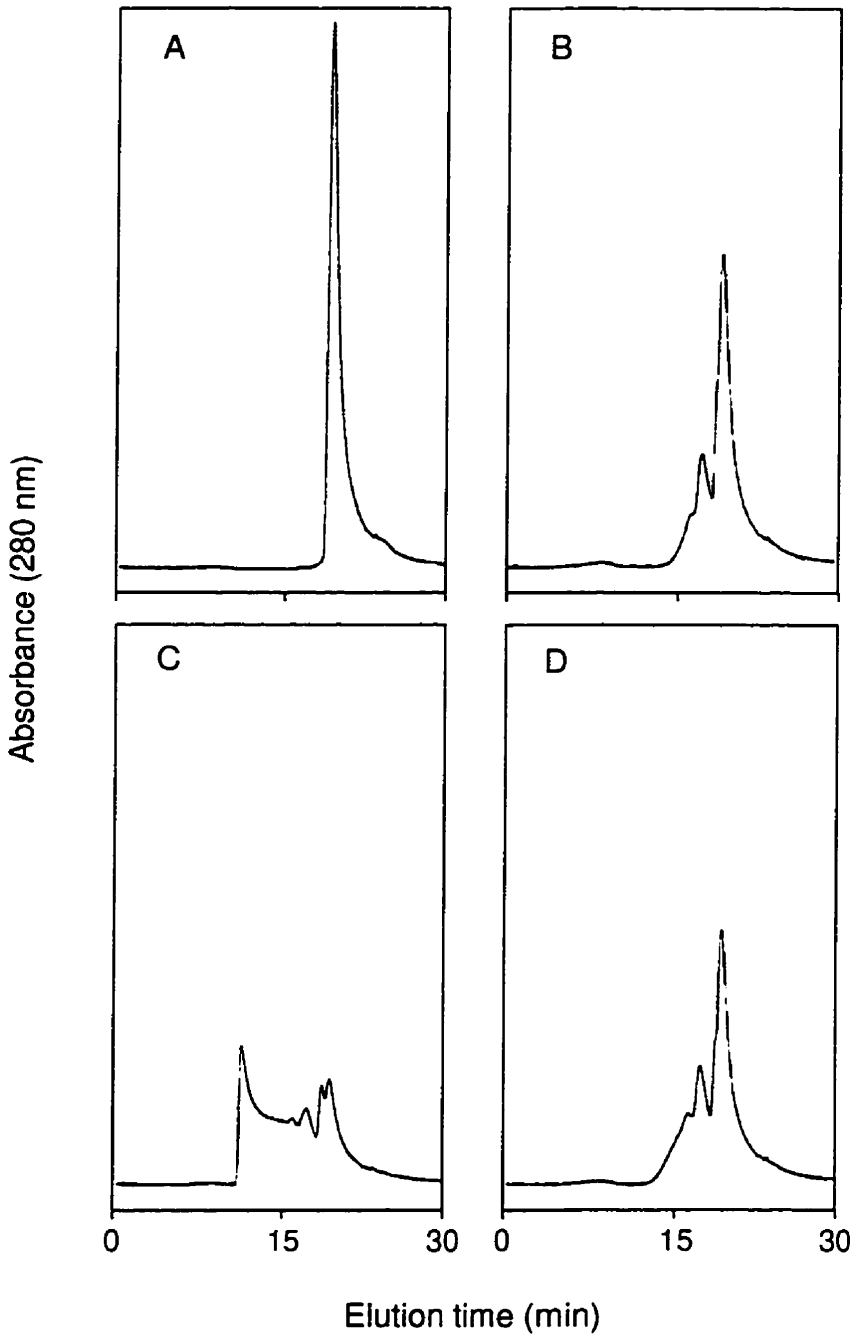

Figure 3.4 Molecular weight distribution analysis of native and polymerized hemoglobin using gel filtration-HPLC with a BioSep Sec-S-3000 column (exclusion limits 5,000-700,000 kD). Representative elution profiles of (A) native hemoglobin, SOD, and catalase, (B) PolyHb-SOD-CAT (8:1 glut:Hb), (C) PolyHb-SOD-CAT (16:1 glut:Hb), (D) PolyHb-SOD-CAT (8:1 glut:Hb) ultrafiltered through 100 kD cutoff membranes.

Figure 3.5 shows the circulation time of superoxide dismutase. Native SOD is rapidly cleared from circulation ($t_{1/2}$ ~ 8-10 min). PolyHb-SOD-CAT (8:1) shows a greater retention of SOD activity with an apparent biphasic clearance pattern. The rapid initial removal phase is likely related to unbound SOD or small SOD-containing oligomers. Greater retention time is achieved following ultrafiltration of PolyHb-SOD-CAT, and polymerization with 16:1 glutaraldehyde/Hb. Ultrafiltration removes some unbound SOD (mw 31,000), and higher polymerization increases the amount of bound SOD.

Figure 3.6 shows the circulation of catalase. Native catalase is cleared rapidly ($t_{1/2}$ ~ 20 min). PolyHb-SOD-CAT (8:1) shows a greater retention of CAT activity. Ultrafiltration of PolyHb-SOD-CAT (8:1) through 100 kD membranes does not remove unbound catalase (mw 232,000), and we found no difference in retention time compared to the original solution. Greater polymerization (16:1) increases the fraction of bound CAT as evidenced by the increase in retention time.

Figure 3.7 represents the circulation time of hemoglobin. Native hemoglobin shows a half-life of approximately 60 min. A greater circulation time is achieved following polymerization (8:1 glut:Hb). Ultrafiltration does not increase retention time compared to non-filtered solution. This is supported by gel filtration analysis which shows that ultrafiltration protocol does not remove significant fraction of tetrameric hemoglobin. Greater polymerization (16:1 glut:Hb) increases the fraction of higher molecular weight hemoglobin molecules as shown by increased circulation time.

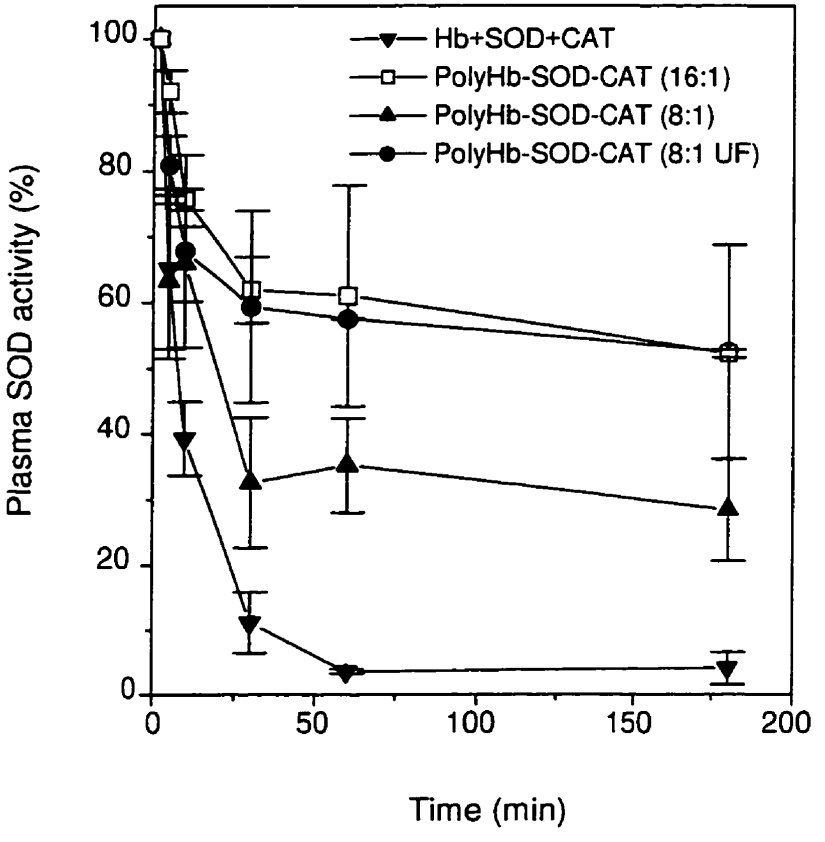

Figure 3.5 Plasma circulation half-life of superoxide dismutase in rats following injection of indicated solutions at a dose of 300 mg Hb/kg animal.

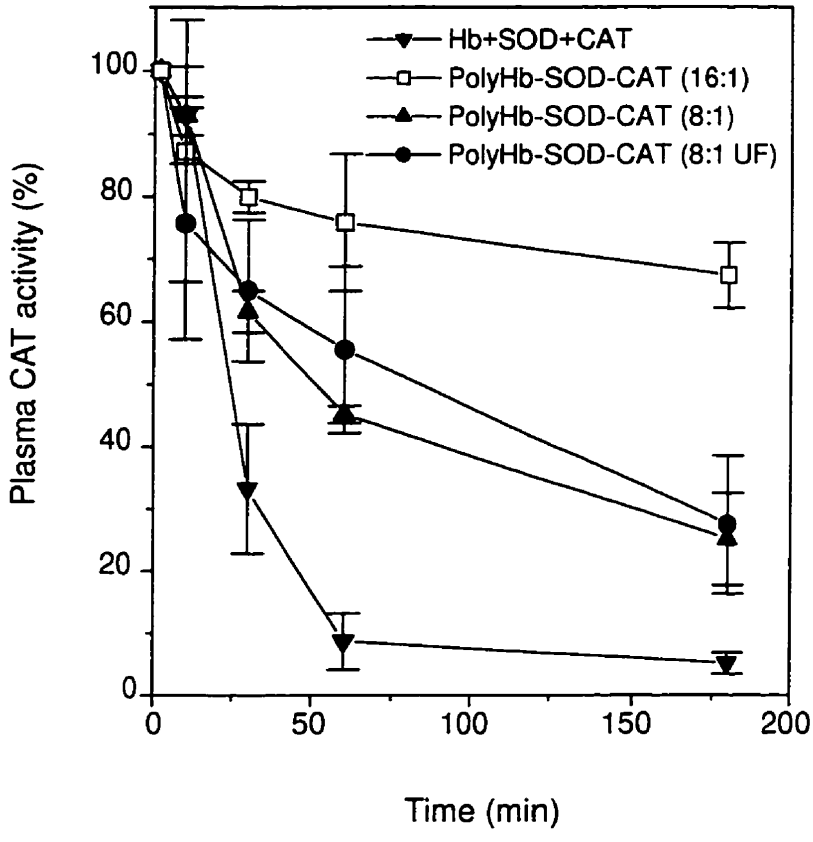

Figure 3.6 Plasma circulation half-life of catalase in rats following injection of indicated solutions at a dose of 300 mg Hb/kg animal.

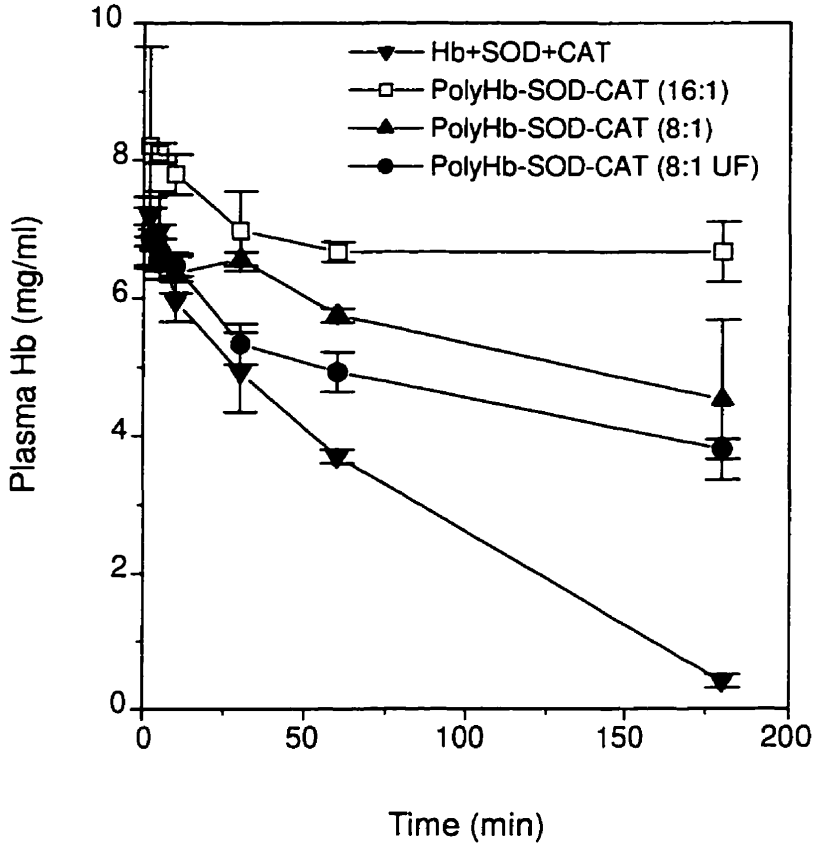

Figure 3.7 Plasma circulation half-life of hemoglobin in rats following injection of indicated samples at a dose of 300 mg Hb/kg animal.

3.6 Discussion

The results in this study show the potential usefulness of glutaraldehyde in polymerizing hemoglobin, SOD and catalase for the development of a modified hemoglobin oxygen carrier with antioxidant properties. Glutaraldehyde preferentially reacts with ε-amino groups of lysine residues of proteins. The non-specific nature of this polymerization produces monomers, oligomers, and polymers differing in size, enzymatic activity, and protein composition. Glutaraldehyde polymerization of hemoglobin has been studied extensively [2,3,36]. Although there are lingering concerns regarding the instability of glutaraldehyde in vivo [3], glutaraldehyde-polymerized bovine and human hemoglobin are currently in clinical trials [4]. Mao et al reported the potential use of glutaraldehyde-linked SOD-catalase conjugates alone [37]. Other SOD and catalase modifications aimed at increasing intravascular times and potential therapeutic effectiveness have also been studied [38,39]. It must be emphasized that combining hemoglobin, SOD, and catalase may also be possible with other types of crosslinkers, encapsulation within artificial cells, or other protein engineering techniques.

Our measurements of total SOD and CAT activity do not differentiate between the enzymatic activities of bound and unbound enzyme fractions. The increased circulation half-lives of SOD and catalase in the highly polymerized solution suggest that more SOD and catalase molecules are part of larger complexes that have longer retention times. There were no differences between total SOD and catalase activity between low

and high polymerization products. Based on this data, we speculate that the increased fraction of bound enzymes have largely retained their activity. Detailed analysis and optimization of enzymatic activity contained in individual molecular weight fractions is in progress.

Some factors may favor the use of bovine hemoglobin over human hemoglobin in the development of acellular hemoglobin-based oxygen carriers. The oxygen affinity of human hemoglobin depends on interaction with 2,3 DPG in the red blood cell. Several approaches have been used to decrease the oxygen affinity of human hemoglobin in the absence of 2,3 DPG [1-4]. Bovine hemoglobin differs from human hemoglobin in that chloride anion modulates oxygen affinity as opposed to 2,3 DPG [40,41]. Since bovine hemoglobin has a lower oxygen affinity than human hemoglobin at physiological chloride concentrations, additional oxygen affinity modifications may not be necessary. Several reports have demonstrated the oxygen delivery efficacy of modified bovine hemoglobins with similar oxygenation characteristics [1-4]. The large scale availability and absence of human viruses are also potential advantages with bovine hemoglobin. However, there may be antigenicity and viral concerns associated with the use of bovine protein sources [3].

The results show that the ratio of SOD/catalase activity affects the degree of oxidative protection afforded to hemoglobin. In both oxidation systems, lower SOD/CAT ratios protected hemoglobin from oxidation suggesting hydrogen peroxide reactions are more detrimental to hemoglobin. This protection fulfills the dual role of

maintaining the oxygen-carrying ferrous form of hemoglobin, and preventing the occurrence of potentially harmful hemoprotein-associated free radical species or the release of hemoglobin degradation products [9]. These in vitro observations are directly applicable to formulations of PolyHb-SOD-CAT. The total antioxidant activity (U/g Hb) and ratios studied in these experiments approximate the SOD and catalase levels found in erythrocytes [42,43]. The SOD/catalase levels found in red blood cells provide useful information. However, different antioxidant requirements may exist outside the red blood cell in the context of a modified hemoglobin blood substitute. These particular antioxidant ratios also approximate the ratios we used for PolyHb-SOD-CAT in previous in vivo studies [23,24].

The amount and ratio of antioxidants may also be relevant with regards to the potential use of PolyHb-SOD-CAT in situations of ischemia-reperfusion injury. Although beneficial effects have been observed in several experimental models, the efficacy of SOD and/or catalase treatments in reperfusion injury remains controversial [38]. Some studies suggest that some of these discrepancies are due to the bell-shaped dose response curve for SOD [44]. Similarly, Mao et al. found that low doses of SOD did not prevent reperfusion injury in ischemic hearts, whereas high doses of SOD aggravated injury [37]. They also showed that the combination of SOD and catalase produced highest degree of protection in reperfused ischemic hearts.

Research on modified hemoglobin oxygen carriers has produced several efficacious red blood cell substitutes. Emphasis will be placed on the safety of

hemoglobin-based oxygen carriers especially in the area of free radical toxicity. Pro-oxidant activity of modified hemoglobin is likely mediated by the generation or interaction with reactive oxygen species, such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Crosslinking hemoglobin, SOD and catalase produces a modified hemoglobin oxygen carrier with the significant ability to counter oxidative reactions, and thus may limit free radical-mediated toxicity.

3.7 References

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3.8 Appendix: Superoxide dismutase assay

This assay is based on the reduction of ferricytochrome c by superoxide radical. Reduced cytochrome c has an absorption peak at 550 nm. The xanthine-xanthine oxidase system was used to generate superoxide radical. The amount of xanthine oxidase used should give a reduction rate of 0.25-0.35 $Amin^{-1}$. In the presence of SOD, the rate of cytochrome c reduction is decreased. A value of rate% is calculated by dividing the rate of cytochrome c reduction observed in samples (or standards) by the reduction rate in samples without SOD multiplied by 100. For calibration curve, rate% values for standards are plotted versus ln (SOD activity). One unit of SOD activity is defined as the amount of enzyme that reduces the rate of cytochrome c reduction by 50 %. This equation may be described as follows; Rate% = $a + b$ ln(SOD activity)[1]. When SOD activity equals 1 unit, Rate% is 50, therefore a is equal to 50. The slope b obtained from the calibration curve is used in subsequent sample activity measurements. The assay was found to be linear between 0.2 and 3 U/ml, as previously reported[1].

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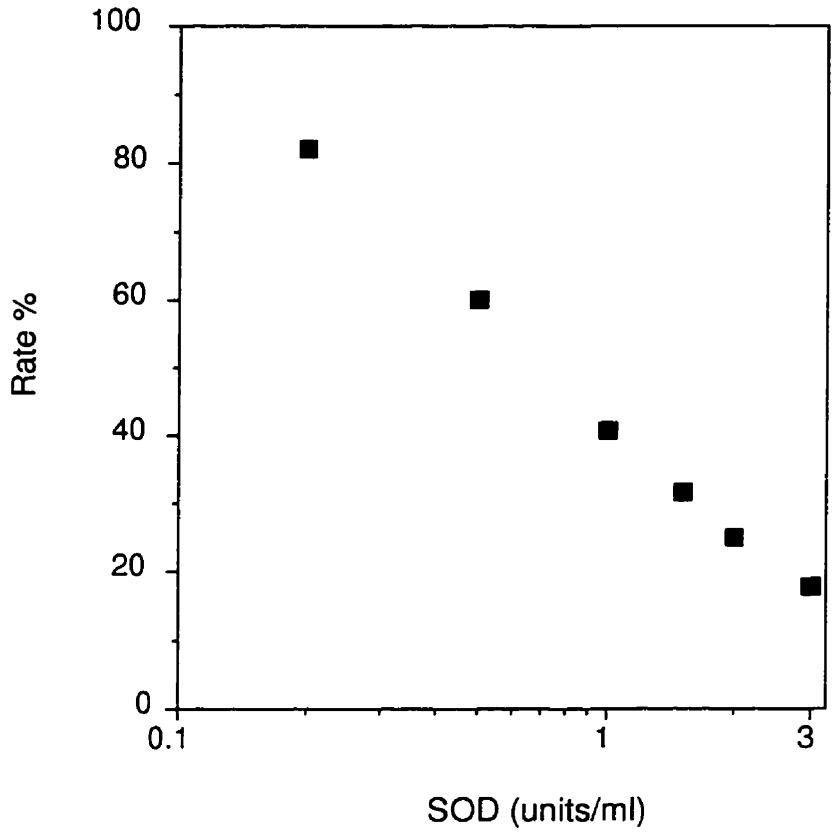

Figure 3.7 Calibration curve for superoxide dismutase activity

3.9 Appendix: Catalase assay

Catalase activity was analyzed by measuring the rate of hydrogen peroxide decomposition at 240 nm[1]. The decomposition of hydrogen peroxide between 10 - 50 mM follows first order kinetics; k = (1/dt) (ln A1/A2) or 2.3/ dt (log A1/A2), where dt represents the time interval of $(t_2 - t_1)$, and A1 and A2 represent the absorbance at t_1 and t_2 respectively. The k constant is used as a direct measure of catalase activity.

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CHAPTER 4

Absence of Hemoprotein-Associated Free Radical Events Following Oxidant Challenge of Crosslinked Hemoglobin-Superoxide Dismutase-Catalase

4.1 Link to previous chapters

In section 1.3, I described the role of hemoglobin in promoting and participating in free radical reactions. In section 1.3.1, I mentioned that superoxide radical and hydrogen peroxide are not reactive per se, however their reactivity is enhanced following interaction with hemoproteins, such as hemoglobin. This is due to the generation of ferrylhemoglobin or hydroxyl radical. These highly reactive species are thought to induce direct molecular damage. One of the central hypotheses of this thesis is that crosslinking SOD and catalase with hemoglobin prevents the production of these harmful hemoprotein-related free radicals in the presence of reactive oxygen species. The purpose of this chapter is to compare the effects of oxidant challenge on PolyHb and PolyHb-SOD-CAT. An in vitro model based on phospholipid peroxidation was used to evaluate the molecular damage resulting from the interaction of hemoglobin and reactive oxygen species. This chapter is also important for establishing the validity of the methods for the measurement of free radicals before planning the in vivo phase of my research.

4.2 Abstract

A potential approach aimed at limiting free radical reactivity of modified hemoglobin red blood cell substitutes involves crosslinking hemoglobin, superoxide dismutase, and catalase (PolyHb-SOD-CAT). In this study, in vitro oxidant challenge experiments were performed with exogenous hydrogen peroxide (H_2O_2) and xanthine oxidase-derived superoxide ($O_2^{\bullet-}$). PolyHb-SOD-CAT was compared to PolyHb for the presence of secondary hemoprotein-free radical events. Absorbance spectra determinations reveal that hemoglobin components are not oxidatively altered in PolyHb-SOD-CAT during oxidant exposure. Ferrylhemoglobin formation, measured as Na_2S-induced absorbance at 620 nm, was prevented with PolyHb-SOD-CAT. Similarly, ferrozine-detectable iron release at high oxidant-heme ratios was inhibited in mixtures containing PolyHb-SOD-CAT. The formation of hydroxyl radical, monitored by salicylate hydroxylation, was also prevented at high oxidant-heme ratios with PolyHb-SOD-CAT. Phospholipid peroxidation was also assessed using thiobarbituric acid assay. Peroxidation was reduced in PolyHb-SOD-CAT mixtures subject to oxidant challenge. Results indicate the effectiveness of PolyHb-SOD-CAT in preventing secondary hemoprotein-associated free radical events. This type of modified hemoglobin oxygen carrier combined with antioxidant activity may reduce the potential toxicity of hemoglobin-based substitutes especially during reperfusion of ischemic tissues.

4.3 Introduction

Hemoglobin is the red blood cell protein responsible for the transport of oxygen. Considerable research has focused on developing crosslinked hemoglobin as potential oxygen-carrying replacement for red blood cells [1-4]. Safe and effective red blood cell substitutes may be advantageous in several clinical settings which require shorter term blood replacement (eg. emergency resuscitation from severe hemorrhage and hemodilution in surgery) [1-4].

Hemoglobin is comprised of four polypeptide subunits, each of which contains a heme active site. Each heme (Fe^{2+}-protoporphyrin IX) can reversibly bind one oxygen molecule. Outside the red blood cell, free hemoglobin breaks down into dimer components that are readily removed from the circulation. Free hemoglobin also has a higher oxygen affinity in the absence of intra-erythrocytic oxygen affinity modulators such as 2,3 diphosphoglycerate (2,3 DPG). A variety of chemical and genetic modifications have been developed to overcome these restrictions [1-4]. Presently, there are several classes of modified hemoglobin undergoing clinical trials in phases one, two, and three [3,4]. The pro-oxidant activity of hemoglobin should be considered in certain applications [5,6].

Hemoglobin can readily generate or interact with free radicals [7]. The spontaneous autoxidation of Fe^{2+}-heme to Fe^{3+}-heme produces superoxide ($O_2^{\bullet -}$) [8]. Superoxide can be reduced spontaneously or enzymatically, via superoxide dismutase,

to hydrogen peroxide (H_2O_2). Catalase and glutathione peroxidase convert H_2O_2 into water and O_2. In the presence of hemoglobin, $O_2^{\bullet-}$ and H_2O_2 increase the rate of methemoglobin formation and may generate highly reactive secondary free radicals such as ferrylhemoglobin or hydroxyl radical. Excessive oxidative damage to hemoglobin leads to the release of iron from heme. In the presence of free iron, $O_2^{\bullet-}$ and H_2O_2 rapidly react to produce hydroxyl radical ($^{\bullet}OH$) (ie. Fenton reaction).

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + O_2$$

It was speculated that intact hemoglobin could catalyze this reaction [9]. However, it is generally believed that this reaction depends on the release of iron from hemoglobin [10,11]. Hydrogen peroxide can also react with hemoglobin to produce ferrylhemoglobin ($^{\bullet}HbFe^{IV}=O$ / $HbFe^{IV}=O$) [12,13]. Ferrylhemoglobin and hydroxyl radical are thought to promote direct cellular injury by reacting with carbohydrates, nucleic acids, and proteins [14,15]. These reactive species can also readily abstract methylene hydrogens from polyunsaturated fatty acids initiating the process of lipid peroxidation.

The antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase help maintain the functional and structural integrity of hemoglobin within the oxidative environment of the red blood cell. Outside the red blood cell, hemoglobin

is free of these protective mechanisms, and thus may undergo oxidative attack by free radicals produced directly from hemoglobin or other endogenous sources. Endothelial cells and circulating neutrophils are especially relevant intravascular sites of free radical generation [16]. There is the significant possibility that modified hemoglobin may aggravate reperfusion injury by supplementing or interacting with oxidants generated following the return of oxygen to ischemic tissue [16,17].

While current hemoglobin modifications confer effective oxygen transport properties to acellular hemoglobin, few approaches have been aimed at limiting the free radical reactivity of hemoglobin. Although adding antioxidants to modified hemoglobin has been suggested in the past [18-20], we proposed the new idea of physically combining antioxidants with hemoglobin [21]. Our design is based on chemically crosslinking SOD and catalase with hemoglobin using the dialdehyde agent, glutaraldehyde [22]. The basic objectives behind this approach are to (1) eliminate free radicals produced by hemoglobin, (2) reduce methemoglobin formation, and (3) prevent oxidative interactions leading to ferryl or hydroxyl radical. Intermolecular polymerization overcomes potential therapeutic restrictions associated with the short circulation times of native SOD and catalase. Indeed, we have shown that glutaraldehyde polymerization increased circulation times of hemoglobin, SOD, and catalase following PolyHb-SOD-CAT administration in rats. Other promising data indicates that reperfusion with PolyHb-SOD-CAT reduces free radical generation compared to polymerized hemoglobin in ischemic isolated hindlimbs and intestine

[23,24]. In this study, we evaluate the effects of in vitro oxidant challenge with $O_2^{\cdot-}$ and H_2O_2 on PolyHb and PolyHb-SOD-CAT. We analyze the production of ferrylhemoglobin, hydroxyl radical, and iron release. We also measure lipid peroxidation using a phospholipid liposome system as an in vitro model of oxidative damage.

4.4 Materials and methods

Materials

Purified bovine hemoglobin was purchased from Biopure Corporation, Boston, MA. Superoxide dismutase from bovine erythrocytes (EC 1.15.1.1, 3300 units/mg stated activity), catalase from bovine liver (EC 1.11.1.6, 48940 units/ml stated activity), xanthine oxidase (from buttermilk) and xanthine were obtained from ICN Biomedicals, Aurora, OH. Sodium salicylate and 2,5 dihydroxybenzoic acid (2,5 DHBA were purchased from Aldrich Chemical Co., Milwaukee, WI. Glutaraldehyde (25%) was obtained from Polyscienes, Warrington, PA. L-α-phosphatidylinositol from bovine liver (10 mg/ml dissolved in chloroform containing mostly arachidonic and steric acids), sodium sulfide (Na_2S), thiobarbituric acid, butylated hydroxytoluene, ferrozine (0.85 %) and iron standard were from Sigma Chemical Co., St. Louis, MO. Hemoglobin assay kit was purchased from Stanbio Laboratory, San Antonio, TX. Hydrogen peroxide (30%), citric acid, sodium acetate and other chemicals were from BDH, Toronto, ON.

Preparation of PolyHb and PolyHb-SOD-CAT

Modified hemoglobin solutions were prepared essentially as previously described [17,19]. Reaction mixtures were prepared containing hemoglobin (7g/dl), SOD (210 U/ml), and catalase (21,000 U/ml) giving a ratio of 1g Hb : 3000 U SOD : 300,000 U CAT. After an initial lysine addition of 10:1 lysine/Hb (mol/mol), glutaraldehyde was added to hemoglobin at a molar ratio of 8:1 glutaraldehyde/Hb. After 3.5 hr at 4 C°

under aerobic conditions with constant stirring, reaction was stopped with the addition of excess lysine at molar ratio of 100:1 lysine/Hb. Solutions were dialyzed in 0.9 % sodium chloride. Hemoglobin concentration was determined by the Drabkin method.

Analysis of spectral changes during oxidant challenge

Reaction volumes were prepared containing 50 μM PolyHb or PolyHb-SOD-CAT (as heme) in 50 mM potassium phosphate, pH 7.4 were incubated with either hydrogen peroxide (500 μM) or xanthine (100 μM)/xanthine oxidase (10 mU/ml). Absorbance spectra (500-700 nm) were recorded at 1 scan/min for 25 min using a Perkin Elmer Lambda B spectrophotometer.

Ferrylhemoglobin measurement

Hydrogen peroxide (5, 50, and 500 μM) was added to reaction volumes containing 50 μM PolyHb or PolyHb-SOD-CAT in 50 mM potassium phosphate, pH 7.4. Xanthine (100 μM)/xanthine oxidase (10 mU/ml) was used as the oxidation system in separate experiments. At given time intervals, excess catalase was added to remove residual hydrogen peroxide in both oxidation systems. Sodium sulfide (2 mM) was added to mixtures, and absorbance was measured at 620 nm. Blank samples consisted of measuring absorbance in similar mixtures except water was added instead of hydrogen peroxide.

Iron release measurement

Hydrogen peroxide was added to PolyHb (50 μM) or PolyHb-SOD-CAT (50 μM) in 50 mM potassium phosphate, pH 7.0. At specified time intervals, reaction was terminated by adding excess catalase. After 2-3 min, ascorbic acid (0.5 ml of 0.02%) was added and mixed for 5 min. Trichloroacetic acid (0.5 ml of 20%) was then added to precipitate proteins. The 1.5 ml mixture was centrifuged, and the supernatant (1 ml) was added to ammonium acetate buffer (0.45 ml) and ferrozine reagent (50 μl). Absorbance of iron colour complex was measured at 560 nm. The amount of iron released was calculated by measuring the absorbance of an iron standard (500 mg/dl), treated as described above, against H_2O blank.

Hydroxyl radical determination

Reaction volumes containing 50 μM of PolyHb or PolyHb-SOD-CAT and salicylate (5 mM) in deionized distilled water were incubated with hydrogen peroxide. At specified reaction times, samples (300 μl) of these mixtures were mixed with 10 μl of catalase stock solution to eliminate residual hydrogen peroxide. These aliquots were centrifuged (14,000 g, 40 min, 4 C°) through Microcon™ microconcentrators (Amicon, Beverly, MA) with 10,000 MW cutoff to remove proteins. Filtrate (40 μl) was directly injected into a HPLC system consisting of an automatic injector (Waters Wisp 712B), solvent delivery system (Waters 6006A), dual-channel integrator (Waters Data Module 746), and an electrochemical detector (Waters 460). Reverse-phase HPLC was carried

Nucleosil guard column. The mobile phase consisted of 30mM sodium acetate-citric acid: methanol (90:10 v/v), and the flow rate was 1 ml/min. The detector potential was set at 0.65 V for the detection of 2,5 dihydroxybenzoate (2,5 DHBA). Standard plots were derived from injections of known amounts of authentic 2,5 DHBA dissolved in mobile phase.

Analysis of phospholipid peroxidation

L-α-phosphatidylinositol liposomes were prepared by evaporating chloroform from the lipid mixture under gentle stream of nitrogen, resuspending dried lipid in 2 ml of 20mM potassium phosphate-150 mM sodium chloride buffer pH 7.4, and sonicating this suspension for 45 sec and vortexing for another 4-5 min. Reaction volumes (0.5 ml) containing liposomes (0.5 mg/ml), PolyHb or PolyHb-SOD-CAT (50 μM as heme) and hydrogen peroxide were incubated at 37 °C. At given time intervals, TCA (0.25ml of 15%) was added, and mixtures were centrifuged for 10 min at 14 000g. Tubes containing supernatant (0.5 ml), TBA (0.25 ml of 0.67%), acetic acid (0.25 ml of 20%) with sodium hydroxide-adjusted pH of 3.5, and BHT (25 μl of 0.2%) were then placed in a boiling water bath for 15 min. After cooling samples, absorbance readings were measured at 532 nm. Results are expressed as malondialdehyde equivalents (nM) based on a calibration curve obtained with 1,1,3,3 tetramethoxypropane.

4.5 Results

Hemoglobin-associated free radical formation

Figure 4.1 shows the result of ferrylhemoglobin formation in the presence of hydrogen peroxide. Ferrylhemoglobin was measured using a method based on the sodium disulfide conversion of ferrylhemoglobin to sulfhemoglobin. This later derivative has an absorbance peak at 620 nm [13]. The addition of hydrogen peroxide to PolyHb produced ferrylhemoglobin (Fig. 4.1A). The amount of ferrylhemoglobin was dependent on the reaction time and concentration of hydrogen peroxide. Figure 4.1B shows the absence of ferrylhemoglobin production with PolyHb-SOD-CAT. The formation of ferrylhemoglobin during incubation with superoxide-generating xanthine oxidase system is shown in Fig. 4.2. In PolyHb samples, ferrylhemoglobin was detected. Ferrylhemoglobin was not detected in mixtures containing PolyHb-SOD-CAT subject to similar xanthine oxidase challenge.

Figure 4.3 represents measurements of ferrozine-detectable iron in PolyHb samples following exposure to hydrogen peroxide. Free iron was detected with the addition of 50 and 500 μM of hydrogen peroxide, while none was found with 5 μM hydrogen peroxide. Iron release was not observed with PolyHb-SOD-CAT at any of the concentrations of hydrogen peroxide. During xanthine oxidase challenge, iron release was not detected in either PolyHb or PolyHb-SOD-CAT.

The hydroxylation of salicylate was used to monitor the formation of hydroxyl radical induced by hydrogen peroxide incubation. The two main hydroxylation products

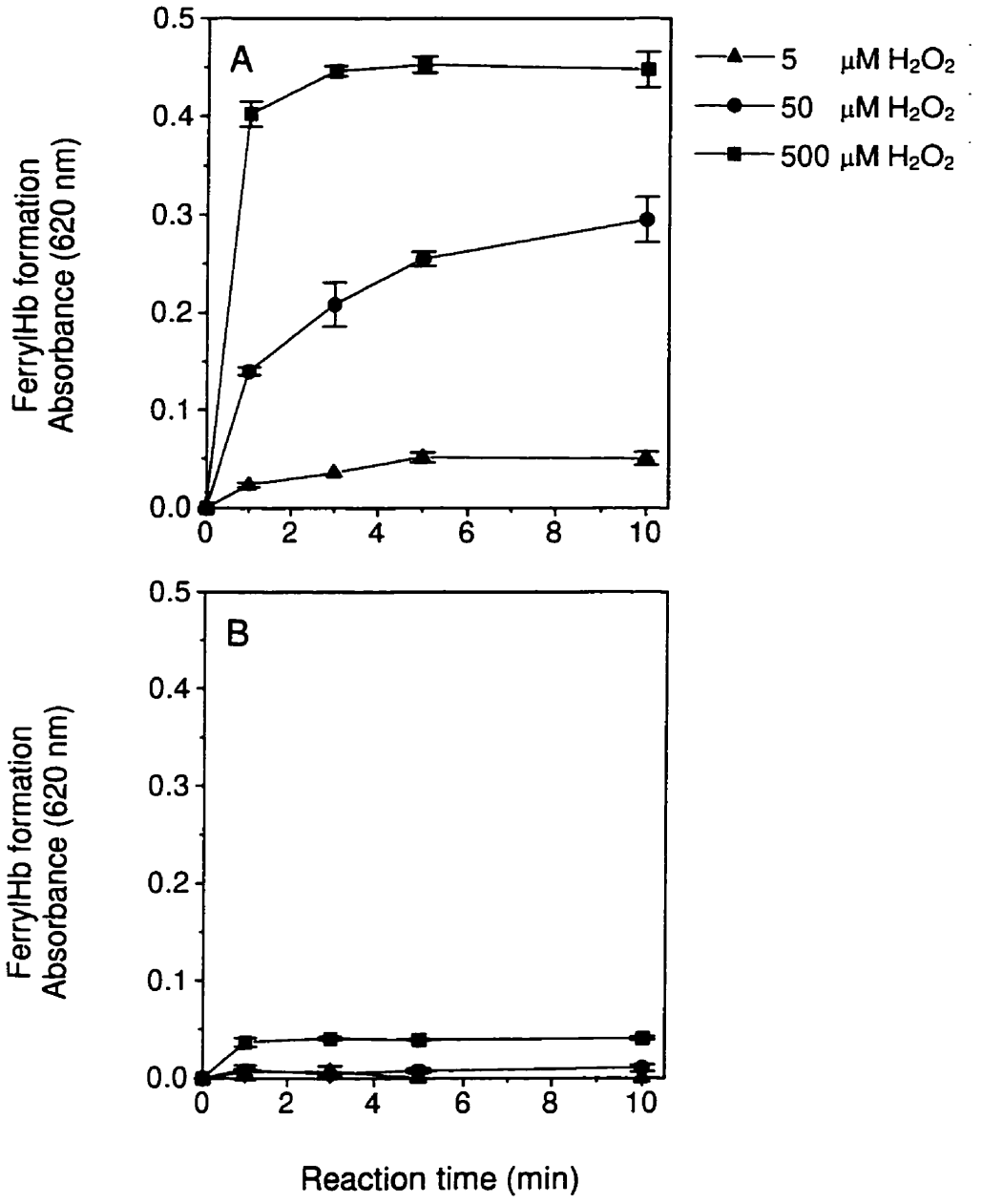

Figure 4.1 Ferrylhemoglobin formation in the presence of hydrogen peroxide (5, 50, 500 μM) in mixtures containing 50 μM of (A) PolyHb and (B) PolyHb-SOD-CAT. Data are plotted as means ± SD of triplicate measurements.

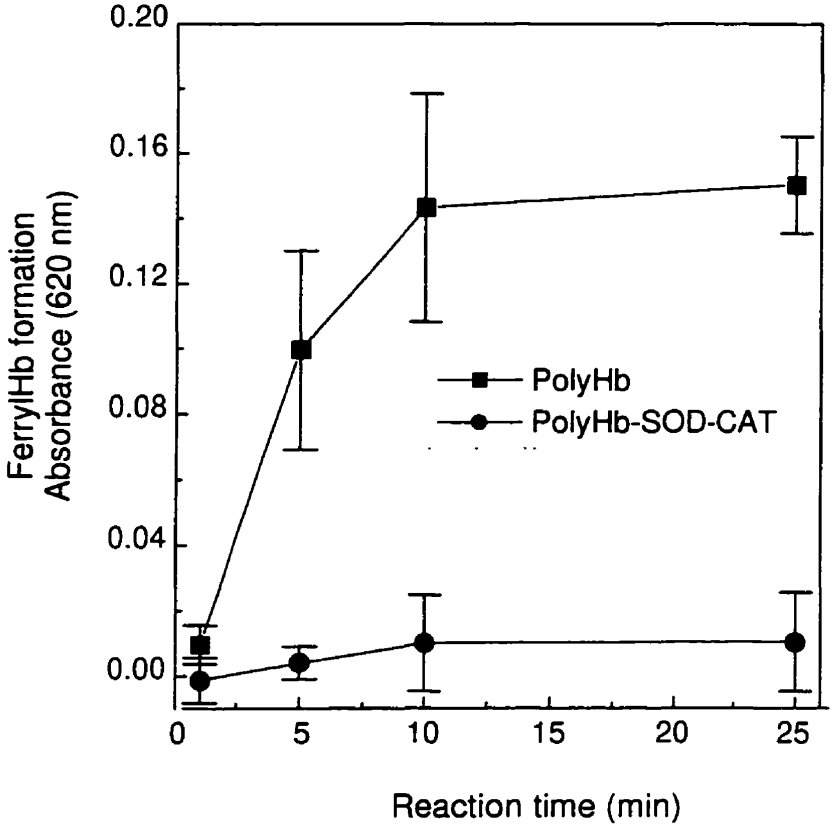

Figure 4.2 Ferrylhemoglobin formation following incubation of xanthine (100 μM) and xanthine oxidase (10 mU/ml) with 50 μM of (A) PolyHb and (B) PolyHb-SOD-CAT. Data are plotted as means ± SD of triplicate measurements.

of salicylate are 2,3 and 2,5 dihydroxybenzoate. Figure 4.4 represents the formation of 2,5 DHBA in reaction mixtures incubated with 500 μM hydrogen peroxide. Hydroxyl radical was detected with PolyHb, whereas none was observed with 5 μM hydrogen peroxide. Increased 2,5 DHBA was also found at 45 min with 50 μM hydrogen peroxide (data not shown). 2,5 DHBA was not detected in PolyHb-SOD-CAT samples. Similarly, previous studies have shown that excess hydrogen peroxide concentrations and long incubation times are needed to generate hydroxyl radical [11].

Phospholipid peroxidation

Figure 4.5 represents the peroxidation of phospholipid, assessed via thiobarbituric reactive substances, generated in mixtures containing liposomal membranes and modified hemoglobin during hydrogen peroxide challenge. Significant peroxidation was measured in PolyHb mixtures containing 500 and 50 μM hydrogen peroxide. At 500 μM hydrogen peroxide, peroxidation was significantly inhibited in reaction mixtures containing PolyHb-SOD-CAT. It was also noted that peroxidation values were higher in PolyHb samples not treated with hydrogen peroxide compared to PolyHb-SOD-CAT. This suggests that autoxidation of PolyHb alone under these incubation conditions were sufficient to induce peroxidation.

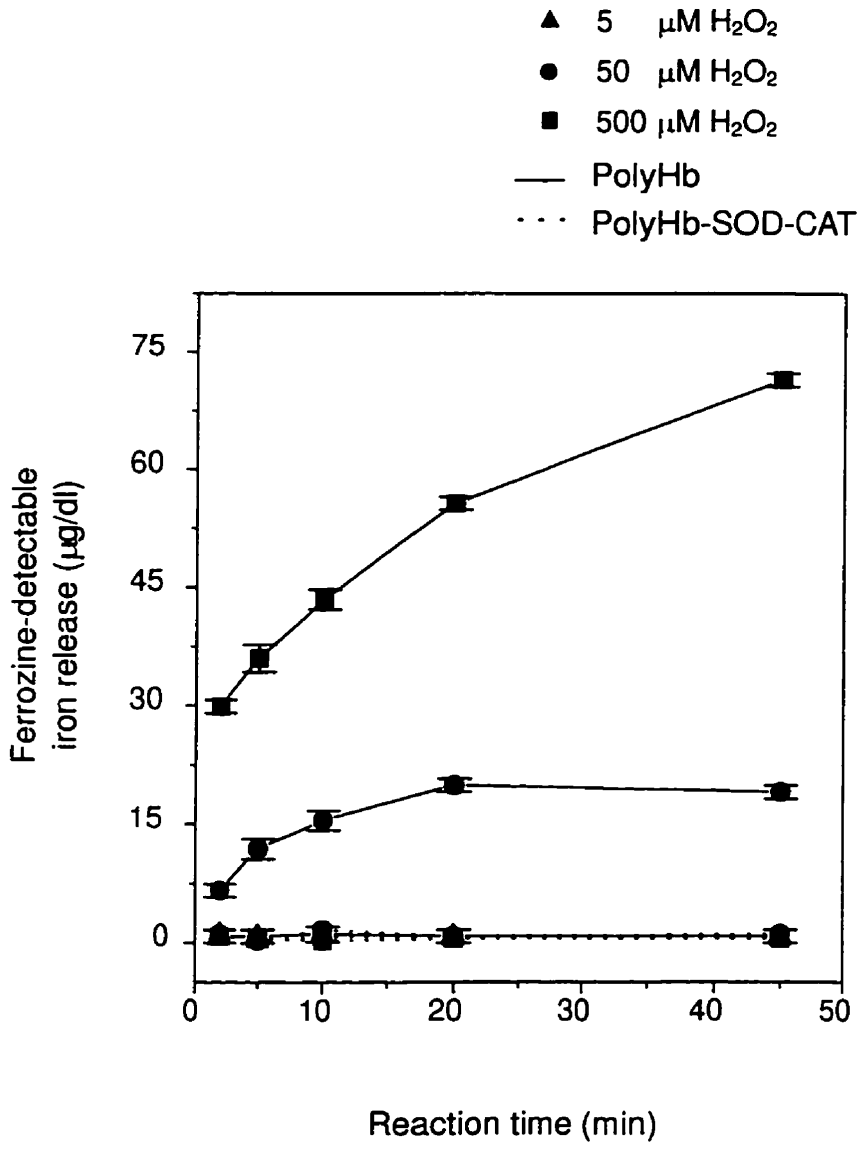

Figure 4.3 Time course of iron release from PolyHb or PolyHb-SOD-CAT (50 μM) in the presence of H_2O_2 (5, 50, 500 μM). Data are plotted as means ± SD of triplicate measurements.

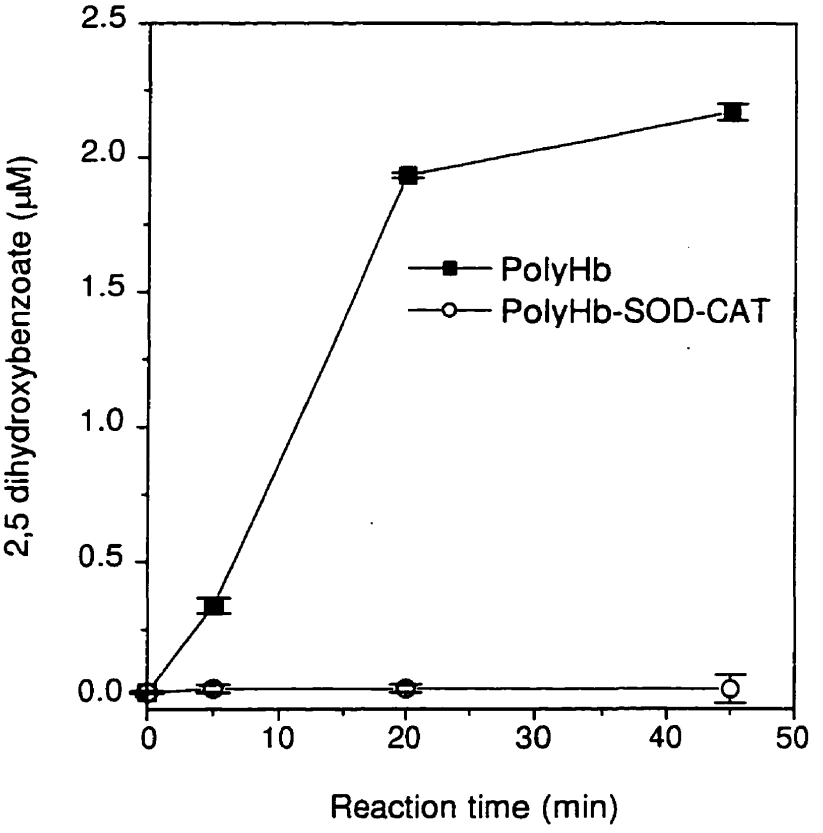

Figure 4.4 Hydroxyl radical formation as measured by the production of the salicylate hydroxylation product, 2,5 dihydroxybenzoate. PolyHb (50 μM) and PolyHb-SOD-CAT (50 μM) were each challenged with hydrogen peroxide (500 μM) in the presence of salicylate. Data represent means ± SD of triplicate determinations.

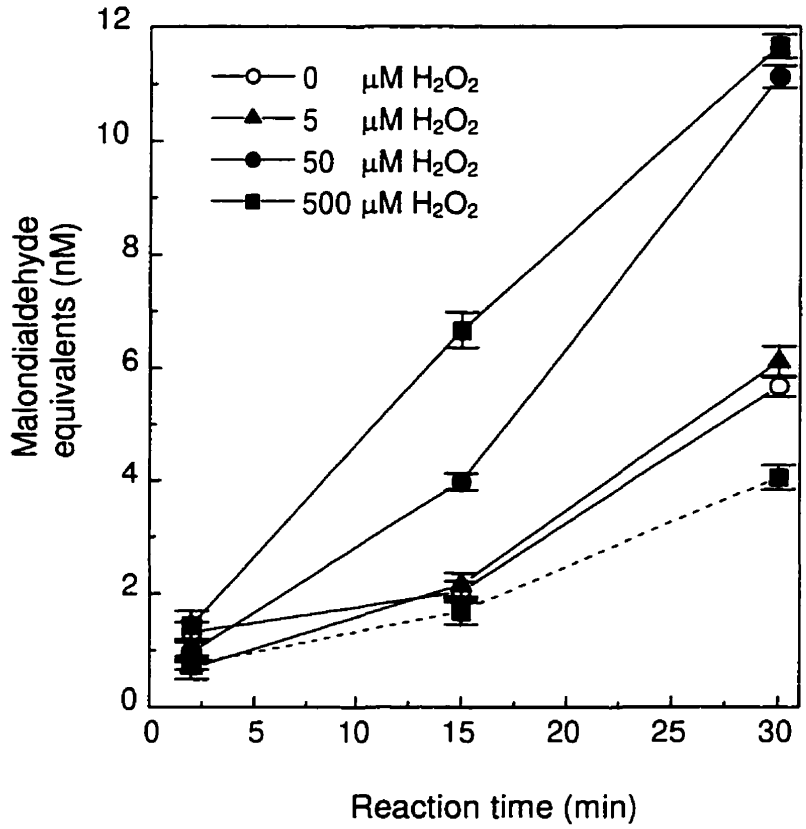

Figure 4.5 Peroxidation of phospholipid liposomes assessed by the thiobarbituric acid assay for malondialdehyde. Hydrogen peroxide was added to reaction mixtures containing liposomes (0.5 mg/ml) and PolyHb (50 μM) or PolyHb-SOD-CAT (50 μM). Data are plotted as means ± SD of triplicate measurements.

4.6 Discussion

Our results clearly show that a modified hemoglobin based on chemically polymerized hemoglobin, superoxide dismutase, and catalase does not participate in hemoprotein-associated free radical events upon oxidant challenge. Since the pro-oxidant effects of hemoglobin are a major safety concern, the present approach constitutes a potential improvement in hemoglobin-based red blood cell substitutes. The antioxidant activity of PolyHb-SOD-CAT may be especially beneficial in situations of increased oxidative stress, such as ischemia-reperfusion injury.

Considerable research interest has focused on the interactions of hemoglobin with superoxide and hydrogen peroxide [7]. Hydroxyl radical and ferrylhemoglobin are highly reactive species produced during oxidant-hemoprotein interactions. Both oxidants are thought to induce direct cellular damage, and are implicated in hemoglobin-induced lipid peroxidation processes [10,18,28-31]. When hemoglobin is incubated in the presence of oxidants that degrade heme and release iron, hydroxyl radical is generated. A recently proposed mechanism also shows that intact hemoglobin can promote the formation of hydroxyl radical by reducing ferric iron metal complexes [32]. These reduced metals can then promote the Fenton reaction.

It is well documented that auto-oxidation of hemoglobin produces methemoglobin and superoxide [8]. Superoxide directly and indirectly, via hydrogen peroxide, leads to further oxidation of hemoglobin and other surrounding molecules. Furthermore, methemoglobin is less stable and more likely to release catalytic forms of iron [33].

Fortunately, under normal circumstances, hemoglobin is safely enclosed within the rich antioxidant milieu of the red blood cell, and methemoglobin reductase reconverts methemoglobin to the ferrous state. Alternatively, Giulva *et al* also described a recycling reaction that may be present in erythrocytes such that ferrylhemoglobin can be converted to methemoglobin in the presence of high amounts of ferrous hemoglobin [12]. Previous work from our laboratory shows that PolyHb-SOD-CAT has a reduced auto-oxidation rate [34]. Plasma is also equipped with sequestering systems for the efficient elimination of various circulating hemoglobin components. These sequestration systems (haptoglobin, hemopexin, ceruloplasmin, transferrin and albumin) also fulfill an antioxidant role by preventing Fenton-like reactions [35,36].

Endothelial cells are highly susceptible to the cytotoxic effects of hemoglobin [37-39]. Balla *et al* showed that the release of heme from methemoglobin induces endothelial damage [38]. They suggest that activated polymorphonuclear cells potentiate this effect by oxidizing hemoglobin to methemoglobin. It is also noteworthy that hemoglobin and modified hemoglobin have been shown to stimulate NADPH oxidase activity of phagocytes [20]. This effect may also be important in the context of ischemia-reperfusion syndromes since neutrophil infiltration and activation is thought to play an important role in the pathogenesis of injury [40]. The cytotoxicity of the ferryl species in endothelial cultures has also been studied [41]. There is also evidence suggesting that endothelial cells increase their release of superoxide and hydrogen peroxide following periods of hypoxia-reoxygenation [42].

Several studies also suggest that in vivo toxicity of hemoglobin is mediated through free radical processes. Simoni *et al* showed that exchange transfusion with modified bovine hemoglobin solution increased lipid peroxidation in heart, liver and plasma [43]. Histological changes to liver and kidney following administration of α-crosslinked bis(3,5 dibrosalicyl)fumarate-Hb (DBBF-Hb) may also implicate lipid peroxidation events [44]. Hemoglobin nephrotoxicity [45] and neurotoxicity [46] has been attributed to heme- and iron-mediated oxidative mechanisms. Biro *et al* observed increased salicylate hydroxylation, indicative of hydroxyl radical formation, and plasma free iron following resuscitation of hemorrhaged dogs with stroma-free hemoglobin [47].

Other attempts directed at reducing the pro-oxidant effects of hemoglobin-based preparations are also emerging. Alayash and coworkers speculate that β-chain stabilization may be a key element in reducing oxidative reactivity. They showed that α-crosslinked DBBF-Hb was more likely to form ferryl radical upon hydrogen peroxide exposure than native Hb A. Conversely, β-crosslinked DBBF-Hb exhibited less ferryl radical upon similar oxidant challenge [27]. Similarly, bovine hemoglobin intramolecularly crosslinked with fumaryl-monodibromoaspirin (FMDA), another β-chain modification, showed less auto-oxidation and oxidant interaction than intermolecularly glutaraldehyde-linked bovine hemoglobin [25]. These observations may be related to the absence of increased oxidative stress in ischemic kidneys

reperfused with diaspirin crosslinked hemoglobin [48]. Polynitroxylated hemoglobins which possess antioxidant properties that mimic superoxide dismutase activity are also under investigation [49].

There are other reasons for using the PolyHb-SOD-CAT combination [21]. For example, the efficient scavenging of superoxide in the vicinity of endothelial cells may prevent inactivation of the endogenous vasodilator, nitric oxide. Preventing this interaction also inhibits the generation of the cytotoxic species, peroxynitrite ($ONOO^-$) [50]. Therefore, if hemoglobin-induced superoxide mechanisms contribute significantly to the vasoconstrictive effect observed clinically and experimentally with modified hemoglobins, scavenging superoxide may partly inhibit this effect. Indeed, the existence of EC-SOD (extracellular SOD) located between endothelial cells and smooth muscle implies the importance of superoxide-nitric oxide interactions in the regulation of vascular tone [51]. Recent findings by Stamler *et al* suggest that nitric oxide-hemoglobin interactions in erythrocytes, specifically interaction with thiols groups of β93 cysteine residues, play important role in vascular tone regulation [52]. Alternatively, the development of a therapeutic agent capable of delivering of oxygen and antioxidants simultaneously may also be useful in reducing the intravascular oxidative stress component of reperfusion injury. Recent studies from our laboratory suggest that reperfusion with PolyHb-SOD-CAT reduces hydroxyl radical generation compared to PolyHb in isolated ischemic hindlimbs and intestine [24,25].

Over the years, several chemical and genetic manipulations of hemoglobin have

produced a series of promising red blood cell substitutes. Based on current toxicity issues, it is likely that continued progress will rely on the development of modified hemoglobins with increased oxidative resistance and a lower propensity to generate reactive oxygen species.

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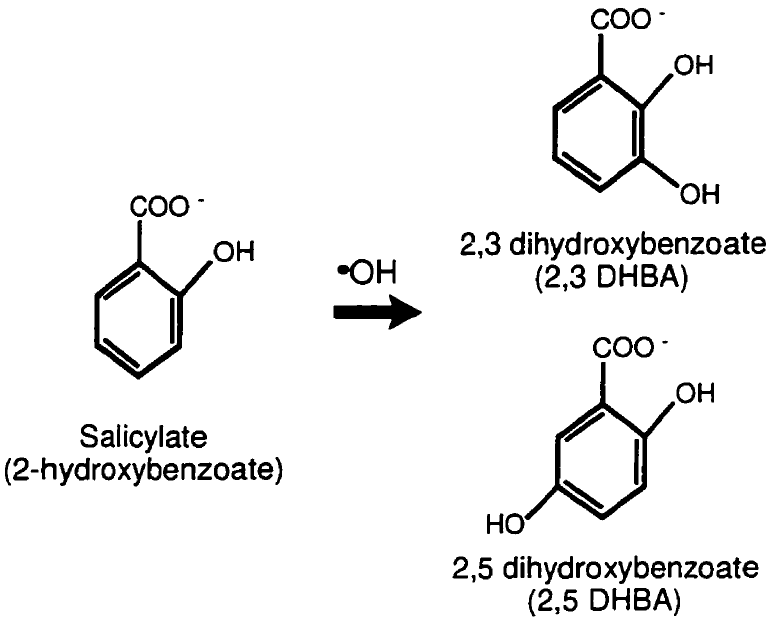
4.8 Appendix: Salicylate as hydroxyl radical trap

The hydroxylation of salicylate is used as an indirect measure of hydroxyl radical formation. The reaction of salicylate with hydroxyl radical generates two main hydroxylation products, 2,3 and 2,5 dihydroxybenzoate (2,3 and 2,5 DHBA). These



products are analyzed by combining electrochemical detection and HPLC reverse phase separation. In my in vitro experiments, I quantified 2,5 DHBA alone, since high levels of 2,3 DHBA were frequently observed in control injections of salicylate alone. The electrochemical detector potential setting that gave maximum response while minimizing background noise was determined by plotting detector response versus potential setting for each standard. At the potential setting used for 2,5 DHBA (0.65 V), salicylate does not exhibit a strong response, and thus cannot be quantified. Alternatively, salicylate can be analyzed by connecting a fluorescence detector in series,

but before the electrochemical detector. The fluorescence detector was set at 313 nm. This allows the measurement of both salicylate and hydroxylation products in the same injection.

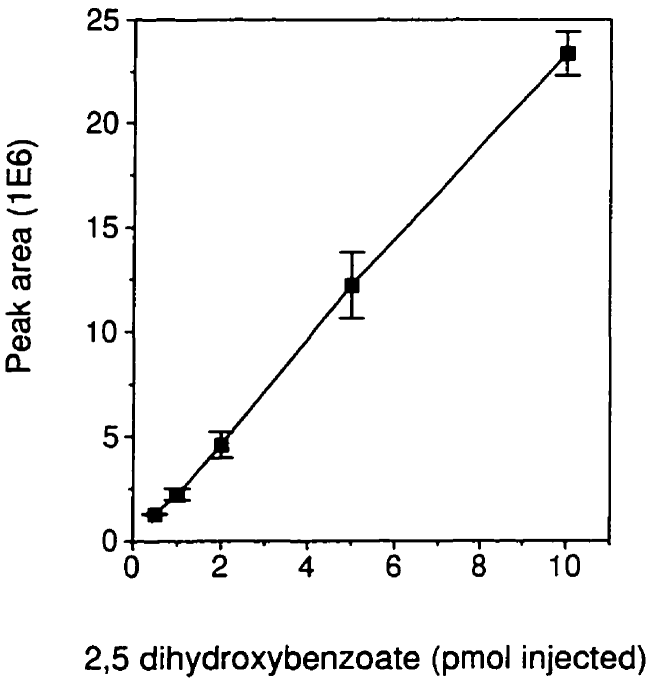


Figure 4.6 Standard curve for 2,5 dihydroxybenzoate using electrochemical detection and HPLC separation.

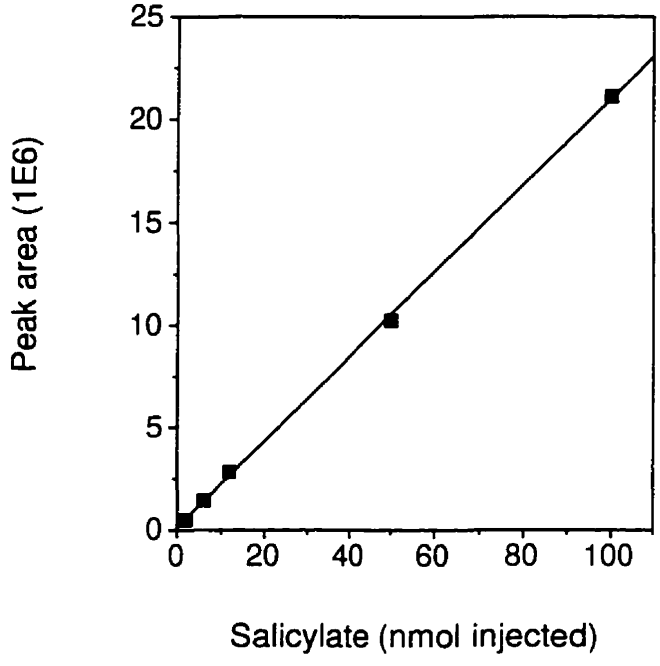


Figure 4.7 Standard curve for salicylate using fluorescence detection and HPLC separation.

4.9 Appendix: Thiobarbituric Acid Test for Lipid Peroxidation

The thiobarbituric acid (TBA) assay is the most popular and easiest method used as an indicator of lipid peroxidation. The assay is based upon the reaction of TBA with malondialdehyde (MDA), an aldehyde product of lipid peroxidation, generating a coloured complex with absorbance peak at 532 nm. Butylated hydroxytoluene (BHT) is included during heating stage to prevent iron-mediated hydroperoxide degradation. Standard curve was obtained by using 1,1,3,3 tetramethoxypropane (1,1,3,3 TMP). This product is hydrolyzed under heat and acid conditions. This standard (0.5 ml of known concentration) was added to test tubes containing TBA (0.25 ml of 0.67%), acetic acid (0.25 ml of 20%) with sodium hydroxide-adjusted pH of 3.5, and BHT (25 µl of 0.2%). These tubes were then placed in a boiling water bath for 15 min. After cooling samples, absorbance readings was measured at 532 nm.

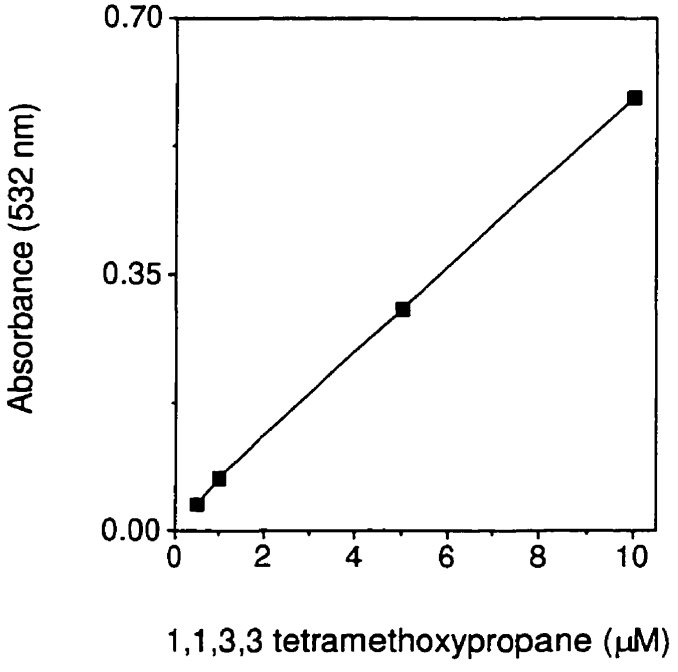


Figure 4.8 Calibration curve for thiobarbituric acid assay using 1,1,3,3 tetramethoxypropane.

CHAPTER 5

Reduction of Hydroxyl Radical Generation in a Rat Hindlimb Model of Ischemia-Reperfusion Injury Using Crosslinked Hemoglobin - Superoxide Dismutase - Catalase

5.1 Link to previous chapters

The in vitro results from the previous chapters clearly indicate that PolyHb-SOD-CAT is effective in preventing oxidative reactions. The purpose of this chapter was to study the role of PolyHb and PolyHb-SOD-CAT in an in vivo model of oxidative stress. Ischemia-reperfusion injury is thought to be mediated by an increase or burst of free radical production at the onset of reperfusion. In this situation, the pro-oxidant activity of modified hemoglobin may aggravate injury. Previous reports suggest that the free radical mechanisms are active in reperfusion injury to skeletal muscle. This chapter focuses on the study of ischemia-reperfusion in isolated rat hindlimbs. My primary focus was to compare the generation of hydroxyl radical in ischemic hindlimbs reperfused with PolyHb and PolyHb-SOD-CAT. A method based on the aromatic hydroxylation of 4-hydroxbenzoate was used to analyze hydroxyl radical.

5.2 Abstract

The effects of PolyHb (intermolecularly crosslinked hemoglobin) and PolyHb-SOD-CAT (intermolecularly crosslinked hemoglobin, superoxide dismutase and catalase) on the production of hydroxyl radical was studied using a rat hindlimb model of ischemia-reperfusion. Hydroxyl radical generation was assessed by an indirect assay based on the hydroxylation of 4-hydroxybenzoate. The hydroxylation product, 3,4 dihydroxybenzoate (3,4 DHBA), was analyzed by high performance liquid chromatography and electrochemical detection. The identification of 3,4 DHBA was confirmed by analysis of authentic standard and an *in vitro* hydroxyl radical generation system. Ischemia was induced in both hindlimbs by ligation of the infrarenal aorta. After a 4hr ischemic period, hindlimbs were simultaneously perfused with PolyHb-SOD-CAT (5 g/dl) into one limb and PolyHb (5 g/dl) into the other limb via femoral arterial catheters. Each perfusate also contained the hydroxyl radical trap, 4-hydroxbenzoate (5 mM). Femoral venous effluents were analyzed for the presence of the 3,4 DHBA. Data indicates that greater 3,4 DHBA production occurs during PolyHb perfusion as compared to PolyHb-SOD-CAT. These preliminary results show that perfusion with PolyHb-SOD-CAT may alleviate oxidative stress in a model of ischemia-reperfusion.

5.3 Introduction

Chemically and/or genetically modified hemoglobins are being developed as potential red blood cell substitutes [1-4]. Previous animal studies demonstrate the oxygen delivery and volume expansion efficacy of modified hemoglobin, and clinical trials have begun in several centers [1-4]. Current research efforts focus on important safety issues related to hemoglobin and free radical chemistry [5,6].

It is well documented that free hemoglobin, and thus acellular modified hemoglobins, can promote and/or participate in a variety of oxidative reactions [7]. Some relevant mechanisms include the release of superoxide anion ($O_2^{\bullet -}$) following hemoglobin oxidation, aberrant activation of phagocyte respiratory burst function, the potential supply of catalytic iron or heme driving the formation of highly reactive hydroxyl ($^{\bullet}OH$) or ferrylhemoglobin ($Fe^{IV}=O$) radical, and the peroxidation of phospholipids [8-17].

The pro-oxidant potential of modified hemoglobin may be especially relevant in ischemia-reperfusion injury. Reperfusion injury is thought to be mediated by oxygen-derived free radicals generated following the return of oxygen to an ischemic tissue or organ [18-20]. Evidence suggests free radical mechanisms play an important role in skeletal muscle reperfusion injury. These include the detection of reactive oxygen species by EPR spectroscopy, enhanced lipid peroxidation (malondialdehyde, lipid hydroperoxides, conjugated dienes), increased xanthine dehydrogenase conversion to

xanthine oxidase, accumulation of end-purine metabolites, neutrophil infiltration and activation during reperfusion [21-26]. Other studies also reported reduced reperfusion effects with the use of free radical scavengers (SOD, catalase, DMTU), iron chelators (desferoxamine), and inhibitors of free radical production (allopurinol) [27-32]. The use of modified hemoglobins to deliver oxygen under these pre-existing conditions of oxidative stress may further exacerbate or promote oxidant tissue damage.

In red blood cells, the oxidative environment created by hemoglobin is controlled by antioxidants which include superoxide dismutase, catalase, and glutathione peroxidase. Outside this protective milieu, hemoglobin molecules may be highly susceptible to interaction or generation of reactive oxidants. We introduced the concept of chemically crosslinking SOD and catalase with hemoglobin [33]. This approach is designed to detoxify reactive oxidants produced by hemoglobin, and to prevent hemoglobin from interacting with other endogenously produced oxidants. Such interactions generate methemoglobin and possibly lead to the formation of highly reactive hydroxyl or ferryl radicals.

Previously, we showed that PolyHb-SOD-CAT effectively scavenges superoxide and hydrogen peroxide [33]. Crosslinked SOD-CAT also prevented methemoglobin formation and iron release in oxidant-challenged reaction mixtures [33,34]. Recent data show that in vitro oxidant challenge of PolyHb-SOD-CAT does not initiate liposomal phospholipid peroxidation, ferrylhemoglobin conversion, nor hydroxyl radical generation. This report investigates the effects of PolyHb and PolyHb-SOD-CAT

perfusion in a skeletal muscle model of ischemia-reperfusion injury. The primary objective is to compare hydroxyl radical generation by analyzing the aromatic hydroxylation product, 3,4 dihydroxybenzoic acid.

5.4 Materials and methods

Materials

Purified bovine hemoglobin was purchased from Biopure Corporation, Boston, MA. Superoxide dismutase from bovine erythrocytes (3300 U/mg stated activity), catalase from bovine liver (72575 U/ml stated activity were obtained from ICN Biomedicals, Aurora, OH. Glutaraldehyde (20%) was obtained from Polysciences, Warrington, PA. 4-hydroxybenzoate (4HB) and 3,4 dihydroxybenzoate (3,4 DHBA) were purchased from Aldrich Chemical Co., Milwaukee, WI. Hemoglobin assay kit was purchased from Stanbio Laboratory, San Antonio, TX. Other chemicals of reagent and HPLC grade were obtained from BDH, Toronto, ON.

Crosslinked hemoglobin-SOD-CAT preparation

Polyhemoglobin was prepared essentially as previously described [35]. PolyHb-SOD-CAT was prepared containing hemoglobin (7g/dl), SOD (210 U/ml), and catalase (21,000 U/ml) giving a ratio of 1g Hb:3000 U SOD:300,000 U CAT. Following an initial addition of lysine (10 lysine:1Hb mol:mol), glutaraldehyde was added to hemoglobin (8 Glut:1Hb mol:mol). After 3.5 hr at 4 C° under aerobic conditions with constant stirring, the reaction was stopped with the addition of excess lysine (100 lysine:1Hb mol:mol). Solutions were dialyzed (dialysis tubing, Spectra/Por, Spectrum Medical Industries, 12,000-14,000 mw cutoff) for 8-12 hours with multiple dialysate changes. The modified Krebs-Hansleit dialysate consisted of (in mM), NaCl (118.4),

KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (24.9), and glucose (5) prepared in deionized distilled water. The final solution was passed through sterile 0.2 μM filter, and hemoglobin concentration was measured by the Drabkin method.

Hindlimb preparation

Fasted male Sprague-Dawley rats (280-290 g) were obtained from Charles River Canada, St-Constant, QC. Animals were anesthetized by intraperitoneal injection of 65 mg/kg sodium pentobarbital (Somnotol, Becton Dickinson, NJ). Additional anesthetic was administered as required. Body temperature was maintained by a warming blanket. Incisions were performed in both hindlimbs below the inguinal ligament, and femoral vessels were carefully isolated. Polyethylene cannulae were inserted and secured distal to the superficial epigastric branches in the femoral arteries (PE-10, Clay Adams) and veins (PE-50, Clay Adams). Proper vessel access was tested with a small volume injection of heparinized saline (50 IU/ml). Arterial cannulae were connected to peristaltic pumps (Gilson Minipuls II). For perfusion pressure measurements, a sidearm was attached to the arterial line and directed to a Statham P23AA pressure transducer and a Grass 79D polygraph recorder (Grass Instrument Co., Quincy, MA).

Ischemia-reperfusion model

Surgical protocol is shown in Figure 5.1. The abdominal aorta and inferior vena cava were ligated just below the left renal artery. An additional ligation was placed at aorta-iliac bifurcation. Hindlimbs were initially perfused with heparinized saline via the

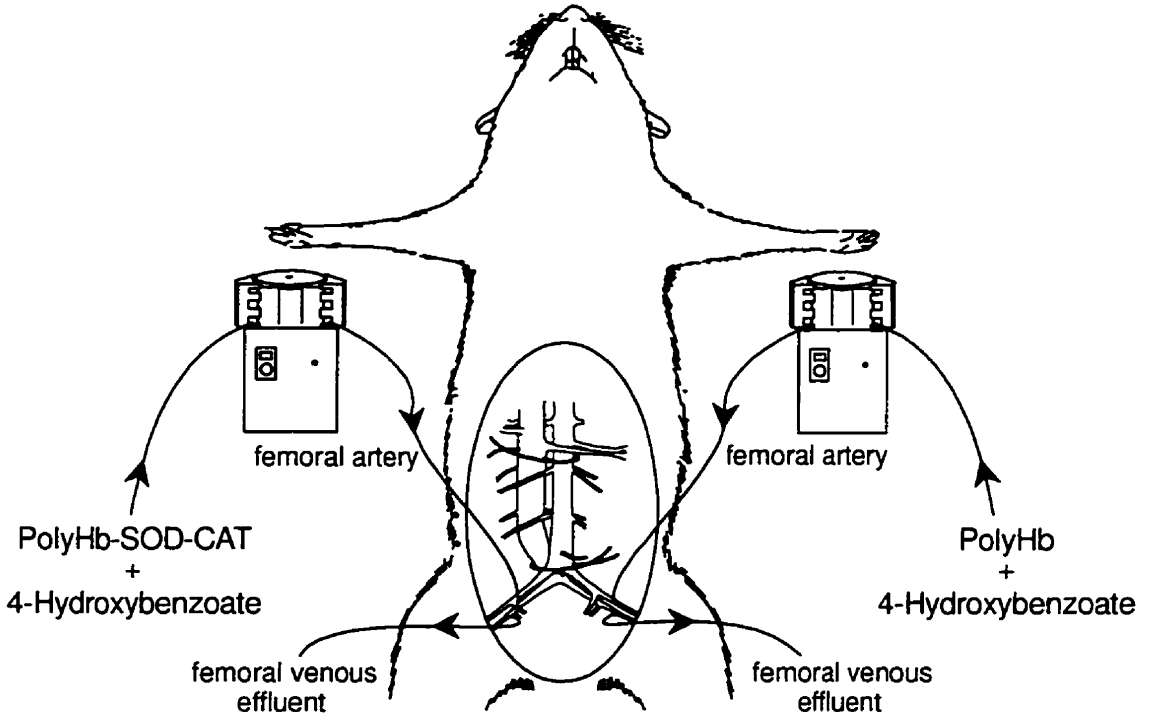


Figure 5.1 Surgical protocol for hindlimb ischemia-reperfusion model.

arterial line to washout blood and ensure flow through venous end. After a 5 min washout period, hindlimbs were subjected to a 4 hour period of no flow. At reperfusion, both limbs were simultaneously perfused at 0.5 ml/min (80-100 mmHg) with two separate modified Krebs-Hansleit baths. One perfusate contained PolyHb (5 g/dl) and the other PolyHb-SOD-CAT (5 g/dl). Three pairs of such studies were carried out. Perfusates were exposed to a gas-liquid interface (95% O_2 - 5% CO_2) with rapid stirring 30 min before reperfusion. Stock solutions of 4-hydroxybenzoate were prepared daily in modified Krebs-Hansleit buffer. Just prior to reperfusion, 4-hydroxybenzoate was added to perfusates at a final concentration of 5 mM. Femoral venous effluents were collected in cold eppendorf tubes, snapped shut, and immediately stored at -80 C°.

Measurement of 3,4 dihydroxybenzoate and 4-hydroxybenzoate

Femoral venous effluents (250 μL) were collected at various times during reperfusion. Aliquots (100 μL) were centrifuged (14,000 g, 40 min, 4 C°) through Microcon™ microconcentrators (Amicon, Beverly, MA) with 10,000 MW cutoff to remove proteins.

Filtrate (20 μL) was directly injected into a HPLC system consisting of an automatic injector (Waters Wisp 712B), solvent delivery system (Waters 6006A), dual-channel integrator (Waters Data Module 746), and an electrochemical detector (Waters 460). Reverse-phase HPLC was carried out on a Nucleosil column, 100 A/ODS, 5μM particle size, 25 x 0.46 cm (CSC, St-Laurent, QC) with Nucleosil guard column. The

mobile phase consisted of 30 mM sodium acetate-citric acid: methanol (90:10 v/v), and the flow rate was 1 ml/min. Detection of 3,4 dihydroxybenzoate (3,4 DHBA) and 4-hydroxybenzoate was performed at an electrochemical potential setting of 0.85 V.

Standard plots were derived from peak area measurements of samples containing known amounts of 3,4 DHBA dissolved in mobile phase. A hydrodynamic voltammogram for 3,4 DHBA was constructed by repeat injections of 3,4 DHBA (10 μL of 10 μM) at different detector potential settings. Positive control experiments were performed by generating hydroxyl radical *in vitro* with mixtures containing xanthine (100 μM), xanthine oxidase (8 mU/ml), $FeCL_3$ (100 μM), and 4-hydroxybenzoate (1 mM) in 50mM potassium phosphate, 0.1 mM EDTA, pH 7.8. In negative controls, the above mixtures also contained SOD (15 U/ml) and catalase (1500 U/ml).

Statistical analysis

Data are expressed as mean ± S.D. Group comparisons of data was done using two-tailed unpaired Student's *t* test. Results were considered significant when $p < 0.05$.

5.5 Results

Analysis of 3,4 dihydroxybenzoate and 4-hydroxybenzoate

Figure 5.2 describes the detector response for 3,4 DHBA versus applied potential. Based on this graph, an electrochemical potential setting of 0.85 V gave best response for 3,4 DHBA while minimizing background noise. At this setting, 4-hydroxybenzoate also exhibited a strong response which allowed its determination in the same isocratic run. Peak identity was confirmed by analyzing effluent samples with and without pure 3,4 DHBA standard. Effluents from perfusion experiments conducted in the absence of 4-HB showed no peaks eluting at the retention times of 3,4DHBA or 4-HB. In samples from an *in vitro* hydroxyl radical system, a peak at eluted at same retention time as the 3,4 DHBA standard. In the presence of superoxide dismutase and catalase, this peak was not detected.

Hydroxyl radical formation in reperfused hindlimbs

Just prior to reperfusion, 3,4 dihydroxybenzoate was not detected in samples collected from PolyHb or PolyHb-SOD-CAT perfusate baths. Figure 5.3 shows an increase in 3,4 DHBA at onset of reperfusion with both PolyHb and PolyHb-SOD-CAT. At 3-5 and 5-7 min, 3,4 DHBA levels were significantly greater in PolyHb compared to PolyHb-SOD-CAT. Figure 5.4 shows the time course of 4-hydoxybenzoate appearance in effluent samples. No significant differences in 4-hydroxybenzoate were noted between time-matched samples in both groups. Figure 5.5 compares the 3,4

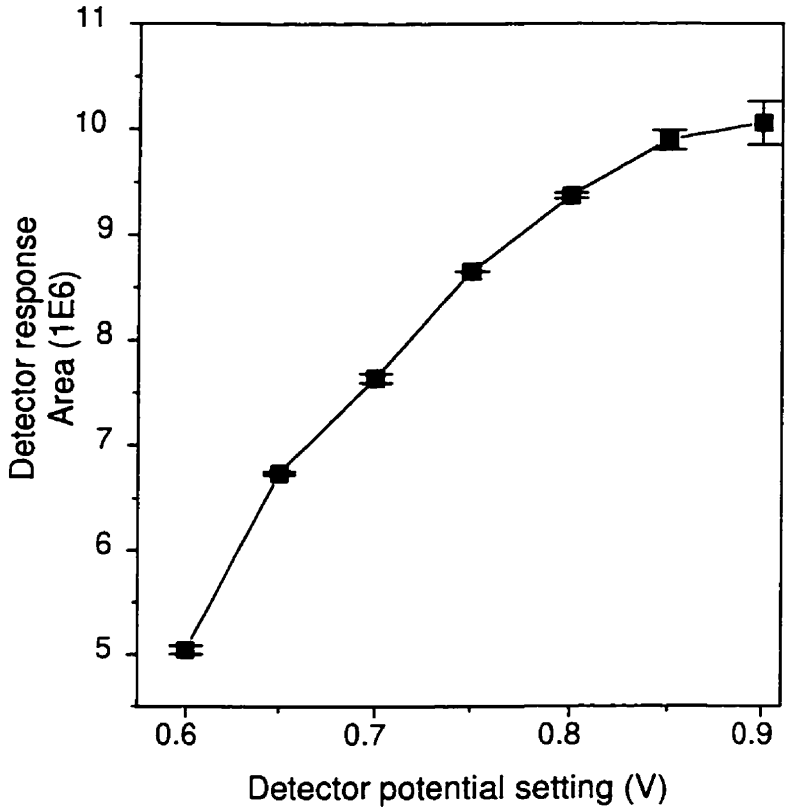


Figure 5.2 Detector response versus electrochemical potential. Reverse-phase HPLC analysis of repeated injections of 3,4 DHBA (10 µL of 1 µM) measured at different potential settings. Solutions of 3,4 DHBA were dissolved in mobile phase containing 30mM sodium acetate-citric acid: methanol (90:10 v/v). Data are presented as means ± SE.

DHBA production between both groups in each animal at all time points and is expressed as a ratio of PolyHb:PolyHb-SOD-CAT. At all time points examined, except for 8-10 min, this ratio is greater than 1 indicating the amount of 3,4 DHBA was greater in PolyHb than in PolyHb-SOD-CAT.

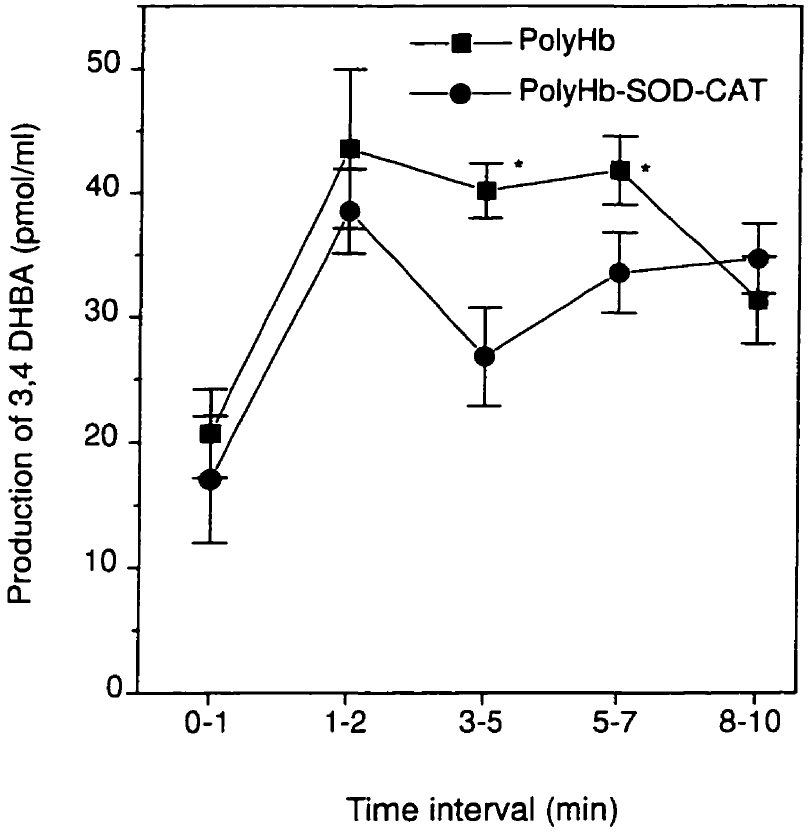


Figure 5.3 Time course of hydroxyl radical production, as determined by the measurement of 3,4 dihydroxybenzoate, in ischemic hindlimbs perfused with PolyHb (■) or PolyHb-SOD-CAT (●). Femoral venous effluents were collected at the start of reperfusion over the indicated time intervals. Samples from these effluents were centrifuged through microconcentrators (10,000 mw cutoff), and filtrates were analyzed for the presence of 3,4 DHBA by HPLC with electrochemical detector potential setting of 0.85 V. Data are presented as means ± SD. * denotes statistical significance, $p < 0.05$.

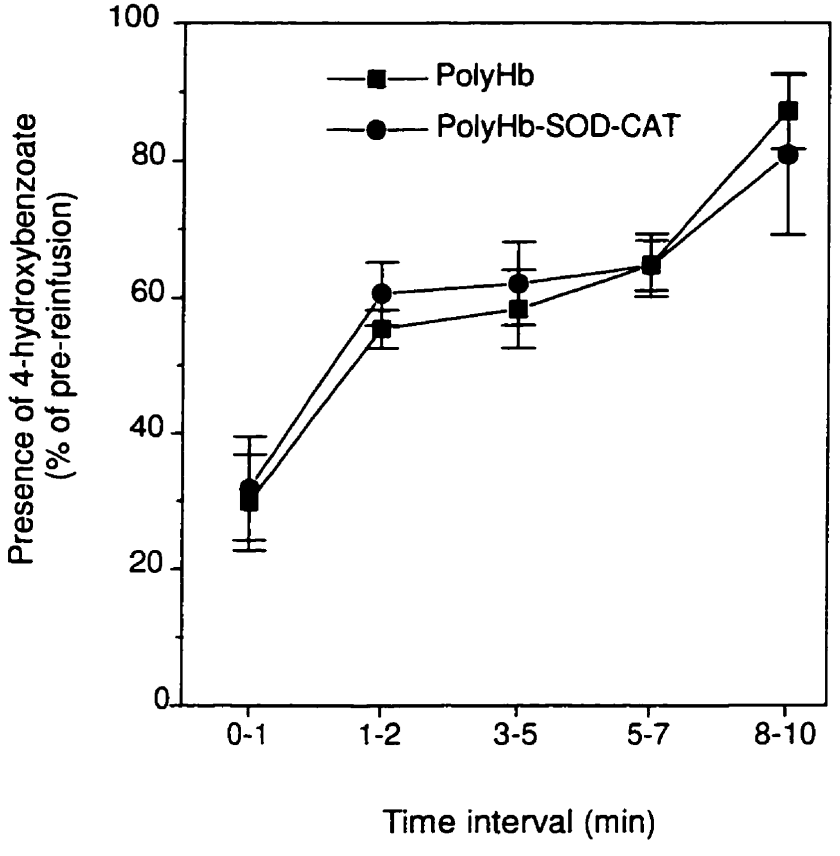


Figure 5.4 The appearance of 4-hydroxybenzoate in effluent samples collected during reperfusion of ischemic hindlimbs perfused with PolyHb (■) or PolyHb-SOD-CAT (●). Values represent % of the pre-reinfusion value. Data are plotted as means of the percentages ± SD.

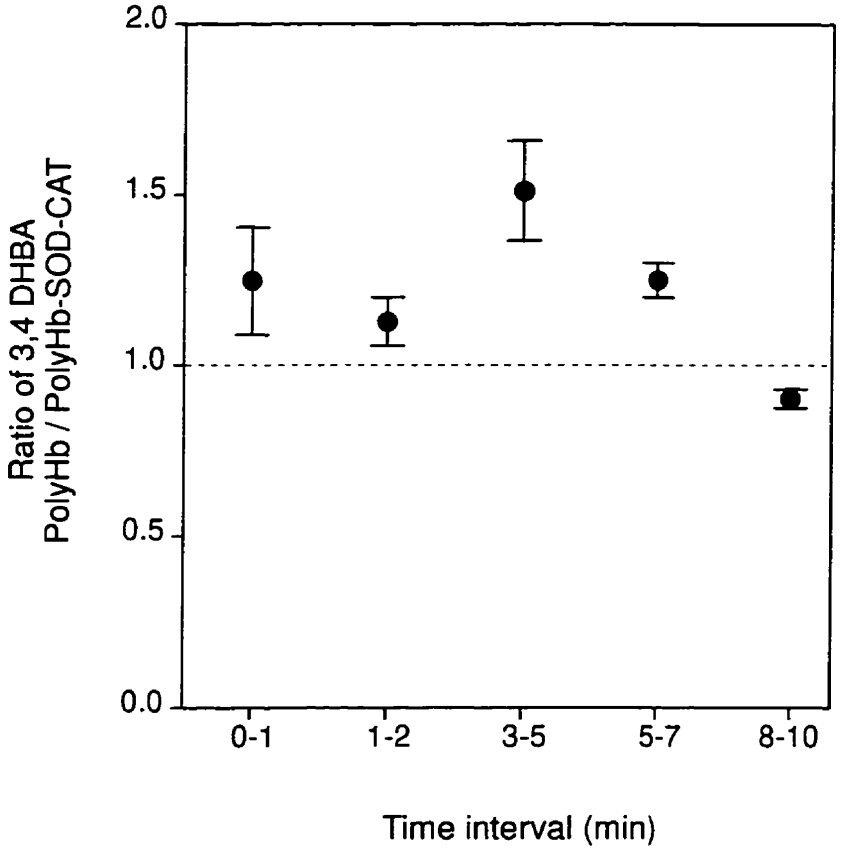


Figure 5.5 For each animal, the 3,4 DHBA levels in the PolyHb- and PolyHb-SOD-CAT-reperfused limbs were compared at each time-matched interval and expressed as a ratio. Plotted values greater than 1 represent time intervals where greater production of 3,4 DHBA was observed in PolyHb compared to PolyHb-SOD-CAT. Values represent means of the ratios ± SD.

5.6 Discussion

Modified hemoglobin oxygen carriers are potential replacement agents for red blood cells. Such hemoglobin-based substitutes, however, may aggravate oxidative stress especially during reperfusion of ischemic tissues. Our recently developed approach based on chemically crosslinking hemoglobin, SOD, and catalase (PolyHb-SOD-CAT) may reduce this pro-oxidant activity. In this study, we investigated the effects of PolyHb and PolyHb-SOD-CAT on the production of hydroxyl radical, measured by the hydroxylation of 4-hydroxybenzoate, in a rat hindlimb ischemia-reperfusion model.

According to the endothelial cell trigger hypothesis, the initial events of ischemia-reperfusion injury occur at the endothelial level [20]. During ischemia, catabolism of ATP leads to the accumulation of hypoxanthine and xanthine, and xanthine dehydrogenase is converted to its superoxide-generating oxidase form. Upon the return of oxygen, endothelial cells produce superoxide via this xanthine-xanthine oxidase pathway. Superoxide is converted spontaneously or enzymatically, via superoxide dismutase, to hydrogen peroxide. In a recent human study, tourniquet application in patients undergoing knee surgery was used as a model of ischemia-reperfusion [36]. After releasing tourniquet (2hr duration), hydrogen peroxide increased and reached a peak at 5 min in blood samples collected from femoral vein. Additional results showing elevated levels of xanthine oxidase, xanthine, and uric acid suggest the partial involvement of the above pathway in free radical generation. Endothelial cells may also

attract and activate circulating neutrophils to the injury site. Activated neutrophils amplify tissue injury by releasing significant amounts of NADPH oxidase-derived superoxide, myeloperoxidase-catalyzed hypoclorous acid (HOCL), proteases, and other tissue degrading products [19]. Other potential free radical sources include defective mitochondrial respiratory chain enzymes, catecholamine autoxidation, and altered arachidonic acid metabolism [20,37].

It has been shown that hemoglobin sensitizes endothelial cells to oxidant damage [38,39]. Balla et al. suggest that this sensitization is mediated by the release of heme from methemoglobin. This strengthens the idea that intravascular oxidation of modified hemoglobin needs to be prevented. Modified hemoglobin can also stimulate phagocytic free radical generation and other inflammatory events [9]. In addition, we have shown that hemoglobin and polyhemoglobin can prime murine peritoneal macrophages for increased superoxide generation (unpublished data). In our study, neutrophil and other blood components were not contributing factors since ischemia and reperfusion phases took place in the absence of blood. Nonetheless, the above considerations strongly support the notion that modified hemoglobin may aggravate existing oxidative conditions in the vasculature during reperfusion. It is conceivable that the delivery of a modified hemoglobin-antioxidant system may alleviate reperfusion-induced oxidative stress at the intravascular level.

In the presence of a metal catalyst, superoxide and hydrogen peroxide rapidly react to form highly reactive hydroxyl radical via classical Fenton chemistry. Although

there has been some debate as to whether hemoglobin-bound iron can drive hydroxyl radical formation [16,17], it is clear that iron released from hemoglobin can generate hydroxyl radical [16]. Recently, another mechanism was proposed by which intact hemoglobin induces hydroxyl radical generation [40]. Biro et al. found that salicylate hydroxylation increased in dogs resuscitated with blood and stroma-free hemoglobin following hemorrhagic shock [41]. The greater hydroxylation observed with hemoglobin resuscitation was attributed to the presence of higher levels of catalytically active iron. Several other endogenous iron sources may become available following ischemia. For example, the iron storage protein, ferritin, can release iron following reaction with superoxide [42]. Muscle myoglobin is another potential iron source in skeletal muscle. Some studies suggest delocalized iron plays an important role in skeletal muscle reperfusion injury [29,30]. In our study, some effluent samples were ultrafiltered, and filtrates were analyzed for the presence of iron using a ferrozine assay with detection limit of 10 μg/ml. Free iron was not detected in these preliminary samples. It is also thought that hydroxyl radical may be produced by a non-iron dependent pathway. The reaction of superoxide and nitric oxide generates another reactive species, peroxynitrite ($ONOO^-$). Peroxynitrite has been shown to decompose to hydroxyl radical at physiological pH [43].

Indirect hydroxyl radical determination based on the hydroxylation of 4-hydroxybenzoate has been described in detail [44]. Other aromatic compounds, mainly salicylate, have been studied in isolated heart, brain, intestine, eye, lung, diaphragm,

and whole body [41,45-51]. In many of these studies, aromatic hydroxylation products appeared within the early minutes of reperfusion consistent with the results reported in this study. Some reports express the need for caution in experimenting and interpreting aromatic hydroxylation assays [52,53]. It is important to exclude hydroxylation produced by non-hydroxyl radical sources or by the manipulation and delivery of the hydroxyl radical trap. In our studies, 3,4 DHBA was not detected in perfusate samples collected directly from the perfusion circuit (pumps and lines) indicating that hydroxylation does not occur during delivery of 4-hydroxybenzoate. Other preliminary studies show that the inclusion of dimethylthiourea (DMTU), a specific hydroxyl radical scavenger, completely inhibits hydroxylation in hindlimbs perfused with PolyHb. In other experiments, 3,4 DHBA was not detected following perfusion of the hydroxyl radical trap alone. This preliminary evidence suggests that 3,4 DHBA is not produced by some endogenous metabolic process. It also suggests that the presence of hemoglobin is required for hydroxylation. Based on the analysis of oxygen saturation curves, PolyHb and PolyHb-SOD-CAT possess oxygen transport characteristics similar to other crosslinked hemoglobins. Although actual oxygen delivery to hindlimbs was not assessed in these studies, favorable oxygen delivery has been observed using a similar limb perfusion protocol in anesthetized dogs perfused with pyridoxylated hemoglobin polyoxyethylene [54]. It is important to determine the degree to which this hydroxylation is actually dependent on the return of oxygen to hindlimbs. Additional experimentation will allow a greater understanding of the individual contributions of

hemoglobin and/or in vivo oxidative processes involved in this hydroxylation event.

Modified hemoglobin research increasingly focuses on issues of free radical reactivity. Development of modified hemoglobin oxygen carriers that incorporate strategies designed to limit their oxidative potentials are likely to gain greater approval in various scientific circles. The results presented in this report suggest decreased oxidant stress following PolyHb-SOD-CAT administration, by indirect assessment of hydroxyl radical formation, in a hindlimb ischemia-reperfusion model. Additional biochemical and physiological studies centered on other measurements of oxidative stress, relevant markers of tissue injury, and indicators of hemoglobin-oxidant interaction will help further determine the significance of these findings.

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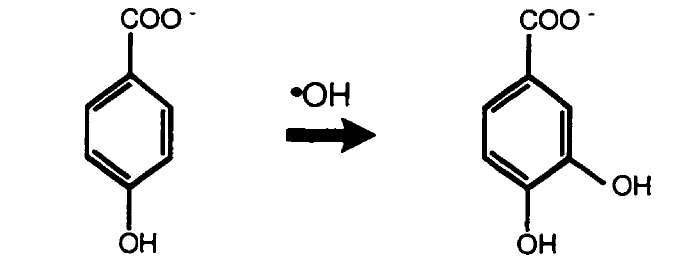
5.8 Appendix: 4-hydroxybenzoate as hydroxyl radical trap

The hydroxylation of 4-hydroxybenzoate is used as an indirect measure of hydroxyl radical formation. The reaction of hydroxyl radical with 4-hydroxybenzoate generates 3,4 dihydroxybenzoate (3,4 DHBA).



4-Hydroxybenzoate

3,4 Dihydroxybenzoate (3,4 DHBA)

Salicylate has by far been the most frequently used hydroxyl radical trap. The aromatic compound, 4-hydroxybenzoate has not received the same attention[1,2]. There are advantages with using 4-hydroxybenzoate. For example, 3,4 DHBA is only main hydroxylation product to quantify. As opposed to the two products formed with salicylate. Furthermore, 4-hydroxybenzoate has same trapping efficiency as salicylate[1]. One of the products of salicylate hydroxylation, 2,5 DHBA, can be generated in large quantities following salicylate injection in vivo. Thus, precautions must been taken in evaluating in vivo data with salicylate. In contrast, very little 3,4 DHBA is produced in systemic circulation following 4-hydroxybenzoate injection in rats[1]. Furthermore, since 4-hydroxybenzoate displays a strong response at the detector potential setting for 3,4 DHBA, both compounds can be measured in the same isocratic run.

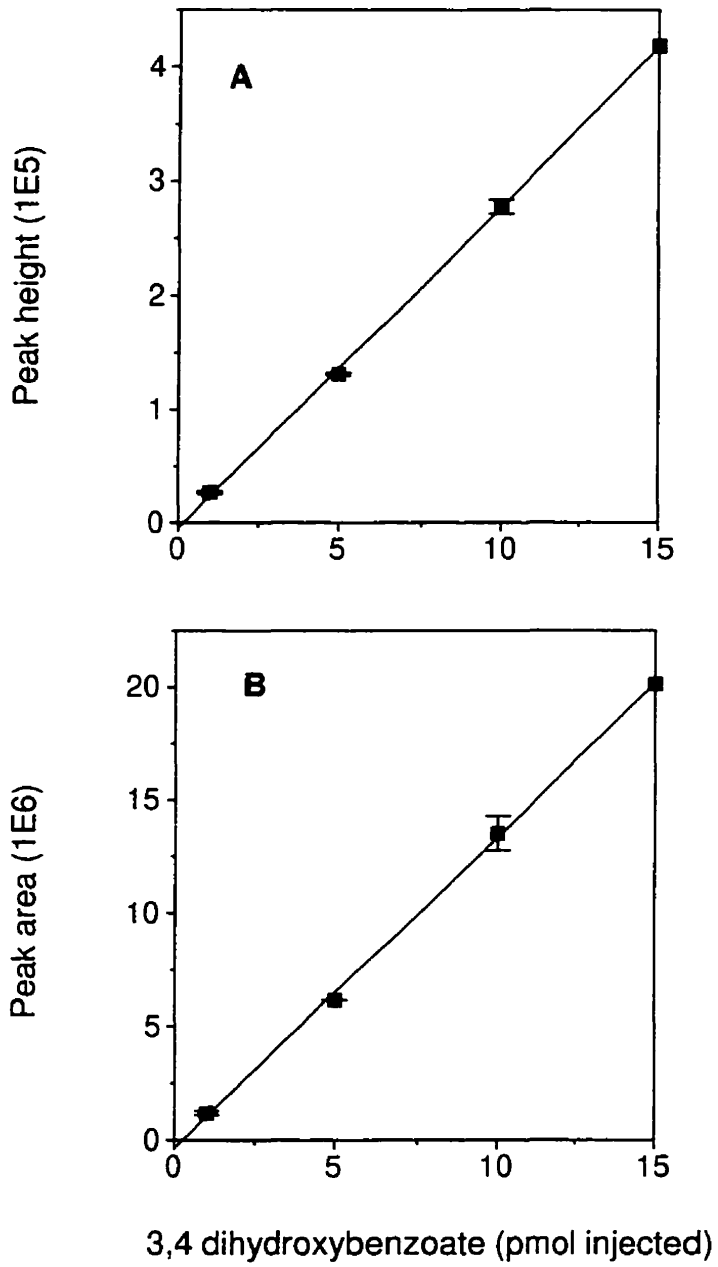


Figure 5.6 Calibration curves for 3,4 dihydroxybenzoate based on (A) peak height and (B) peak area using electrochemical detection and HPLC separation

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2. Ste-Marie L. *et al, Analytical Biochemistry* 241:67, 1996.

5.9 Appendix: In vitro hydroxyl radical generation

Hydroxyl radical was generated *in vitro* in mixtures containing xanthine (100 μM), xanthine oxidase (8 mU/ml), $FeCL_3$ (100 μM), and 1 mM of 4-hydroxybenzoate (4-HB) in 50mM potassium phosphate, 0.1 mM EDTA, pH 7.8. In negative controls, the above mixtures also contained SOD (15 U/ml) and catalase (1500 U/ml). In this system, superoxide radical reduces ferric iron, and also generates hydrogen peroxide by dismutation. Hydroxyl radical is produced via Fenton reaction.

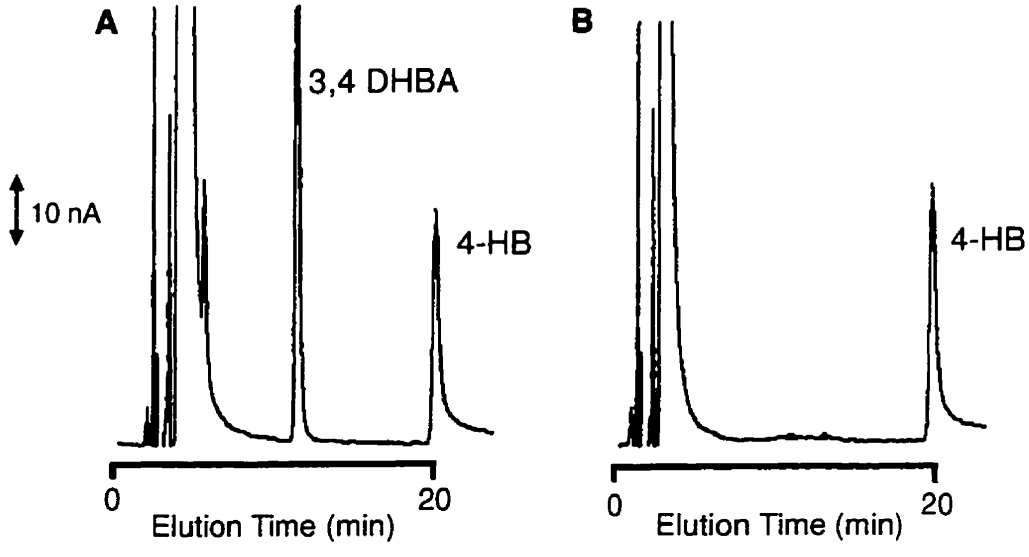

Figure 5.7 (A) Hydroxyl radical generated in mixtures contains xanthine-xanthine oxidase-$FeCl_3$, (B) same mixtures but in the presence of SOD and catalase.

CHAPTER 6

Crosslinked Hemoglobin-Superoxide Dismutase-Catalase Scavenges Free Radials in a Rat Model of Intestinal Ischemia-Reperfusion Injury

6.1 Link to previous chapters

The purpose of this chapter was to study the role of modified hemoglobins in a rat model of intestinal ischemia-reperfusion injury. As in chapter 5, my primary focus was to compare the generation of hydroxyl radical upon reperfusion with PolyHb or PolyHb-SOD-CAT. Similarly, 4-hydroxybenzoate was used as the hydroxyl radical trap. The intestine is particularly susceptible to reperfusion injury due to the presence of high levels of xanthine oxidase.

6.2 Abstract

An *in vivo* rat model of isolated intestinal ischemia-perfusion was developed. This is used to compare the effects of crosslinked hemoglobin (PolyHb) versus crosslinked hemoglobin-superoxide dismutase-catalase (PolyHb-SOD-CAT) on free radical generation in ischemia-reperfusion. Fasted, anesthetized male Sprague Dawley rats underwent midline laparotomy with cannulation of the abdominal aorta and inferior vena cava. Ligation was carried out at the renal pedicles bilaterally and the aorta and vena cava proximally at the diaphragm and distally above the femoral bifurcation. The system was flushed of blood with 20 ml of lactated Ringer's solution. The portal vein was then cannulated with distal clamping at the porta hepatis so that isolated intestinal perfusion could be achieved with the aorta as the inlet and the portal vein as the outlet. Following a 90 minute ischemic time, perfusates containing modified hemoglobin (5 g/dl) and 4-hydroxybenzoate (5 mM) were infused at 0.8 ml/min for 10 min. Portal vein effluent samples were collected at 2.5 minute intervals. Hydroxyl radical generation was assessed by an aromatic hydroxylation technique with 4-hydroxybenzoate (4HB). Reaction of hydroxyl radical with 4HB produces 3,4 dihydroxybenzoate (3,4 DHBA). In the PolyHb group, the levels of 3,4-DHBA increased 10.75-13.58 x-fold above pre-perfusion values compared to 2.25-3.75 x-fold in PolyHb-SOD-CAT group. This indicates that PolyHb-SOD-CAT is effective in reducing *in vivo* hydroxyl radical generation following reperfusion. Since free radicals may play a major role in the pathogenesis of ischemia-reperfusion injury, this suggests a role for PolyHb-SOD-CAT

as a possible protective perfusate in intestinal reperfusion injury.

6.3 Introduction

Crosslinked hemoglobin has been shown to be an effective oxygen carrier and plasma expander in animal models of hemorrhagic shock [1-3]. Concern arises, however, over the possibility of increased oxidative stress due to the exposure of large amounts of heme iron that would normally be sequestered within the erythrocyte [4]. In the presence of iron under physiologic conditions, the hydroxyl free radical ($^{\bullet}OH$) can be created by the metal ion dependent breakdown of hydrogen peroxide (H_2O_2) (the Fenton reaction). $^{\bullet}OH$ is an extremely reactive oxygen-derived free radical and is thought to be a major contributor in oxidative tissue injury. The possible exacerbation of oxidative stress associated with the use of modified hemoglobins led to the development in our laboratory of a crosslinked polyhemoglobin-superoxide dismutase-catalase (PolyHb-SOD-CAT) [5]. In vitro studies show that PolyHb-SOD-CAT effectively scavenges superoxide and hydrogen peroxide [5]. This paper reports one of our in vivo studies on the generation of oxygen radicals comparing PolyHb-SOD-CAT to PolyHb.

Organ reperfusion after a period of ischemia provides a model of *in vivo* oxidative stress [6,7]. Numerous studies have shown that the histological injury observed after a period of ischemia with the re-establishment of organ perfusion can paradoxically be worse than an equivalent period of ischemia alone [8,9]. The presumption is that the injury seen on reperfusion is at least in some way partially oxygen dependent. This oxygen dependent injury is thought to be mediated through the generation the reactive

oxygen species. Several studies have shown that the degree of reperfusion injury was greatly reduced with the use of free radical scavengers such as superoxide dismutase/catalase or dimethylthiourea (DMTU) [10,11]. It is known that a potential source of free radicals occurs via the abnormal catabolism of purines in the reperfused state. Normally, purines are reduced by xanthine dehydrogenase to uric acid. During ischemia, this enzyme is converted to the xanthine oxidase form at a rate proportional to the duration of ischemia. Xanthine oxidase catalyzes the formation of superoxide ($O_2^{\cdot-}$) as oxygen is returned with reperfusion. Use of the xanthine oxidase/dehydrogenase inhibitor allopurinol has been shown to attenuate free radical generation during reperfusion after a period of ischemia, which offers evidence for the role of this enzyme in the generation of free radicals in reperfusion injury [12]. The intestine is a particularly rich source of xanthine dehydrogenase/oxidase and is known to be particularly vulnerable to ischemia-reperfusion injury.

We have developed a model of isolated intestinal perfusion in the rat in order to study the effect on free radical generation of reperfusion with PolyHb and PolyHb-SOD-CAT after a period of ischemia. It was hypothesized that PolyHb-SOD-CAT would show reduced free radical generation on reperfusion as compared to the PolyHb. Measurement of hydroxyl radical was accomplished by high performance liquid chromatography separation and electrochemical detection of 3,4 dihydroxybenzoate (3,4 DHBA), the hydroxylation product of 4-hydroxybenzoate (4HB).

6.4 Materials and methods

Materials

Preparation of intestinal ischemia-reperfusion model

Fasted male Sprague-Dawley rats (280-310g) were anesthetized with intraperitoneal injection of pentobarbital (Somnotol, 65 mg/kg). This was followed by midline laparotomy. The left and right renal pedicles were mobilized, isolated and ligated. The abdominal aorta (Ao) was isolated and cannulated just proximal to the bifurcation by catheter-over-the-needle technique (24 gauge angiocath, Critikon, Tampa, Fl) and fixed in place by ligature of the aorta and vena cava distal to the catheter insertion. The abdominal inferior vena cava (IVC) was then isolated just proximal to this ligation and cannulated and affixed in a similar manner with a 22 gauge angiocath. At this point mid-line sternotomy was performed and the thoracic IVC and descending aorta were isolated and ligated, whereupon the animal died. Intestinal ischemia was taken to begin at this point. A closed circuit for intestinal and liver perfusion was created with the aortic cannula as the inlet and the IVC cannula as the outlet.

The system was flushed with 20 ml of heparinized (10 units/ml) lactated Ringer's solution delivered by peristaltic pump at 0.8 ml/min. This flow rate was chosen because early feasibility experiments revealed that at this rate the pressures generated in the aorta was in the range of 80-90 mm Hg (mean). The circuit remained static for the rest of the ischemic time (90 minutes total). During the time of laparotomy, the intestines are kept moist with lactated Ringer's soaked gauze.

After flushing, the portal vein was isolated and cannulated with a 20 gauge angiocath and fixed in place by ligature of the vessel. Portal vein cannulation was performed at this point since it was found that when the portal vein was cannulated prior to flushing, there was an unacceptably high rate of portal venous thrombosis which rendered the animal unsuitable for experimentation. A small amount of heparinized lactated Ringer's solution (0.2-0.4 ml) was delivered via the aortic cannula to fill the dead-space of the cannula with fluid (Fig. 6.1).

After the 90 minute ischemic time, PolyHb or PolyHb-SOD-CAT (5 g/dl) containing 5 mM of 4HB was perfused at 0.8 ml/min for 10 minutes without recirculation. Portal vein effluent samples were collected, centrifuged, and the spun perfusate was stored at -80 °C for later analysis by high performance liquid chromatography.

PolyHb and PolyHb-SOD-CAT groups consisted of 3 animals each. In addition, 2 control animals were also done: 1 PolyHb animal without 4HB and 1 PolyHb-4HB animal with the addition of 5 mM dimethylthiourea (DMTU) to the perfusate.

Preparation of PolyHb and PolyHb-SOD-CAT

Polymerized hemoglobin solutions were prepared as previously described [5]. PolyHb-SOD-CAT was prepared from starting concentrations of 1 g Hb:3000 U SOD:300000 U CAT.

Analysis of 3,4 dihydroxybenzoate

Quantity of 3,4-DHBA was measured in centrifuged, thawed perfusate samples using HPLC with electrochemical detection as described [13].

Statistical Analysis

Data are expressed as mean ± S.D. Group comparisons of data was done using two-tailed unpaired Student's *t* test. Results were considered significant when $p < 0.05$.

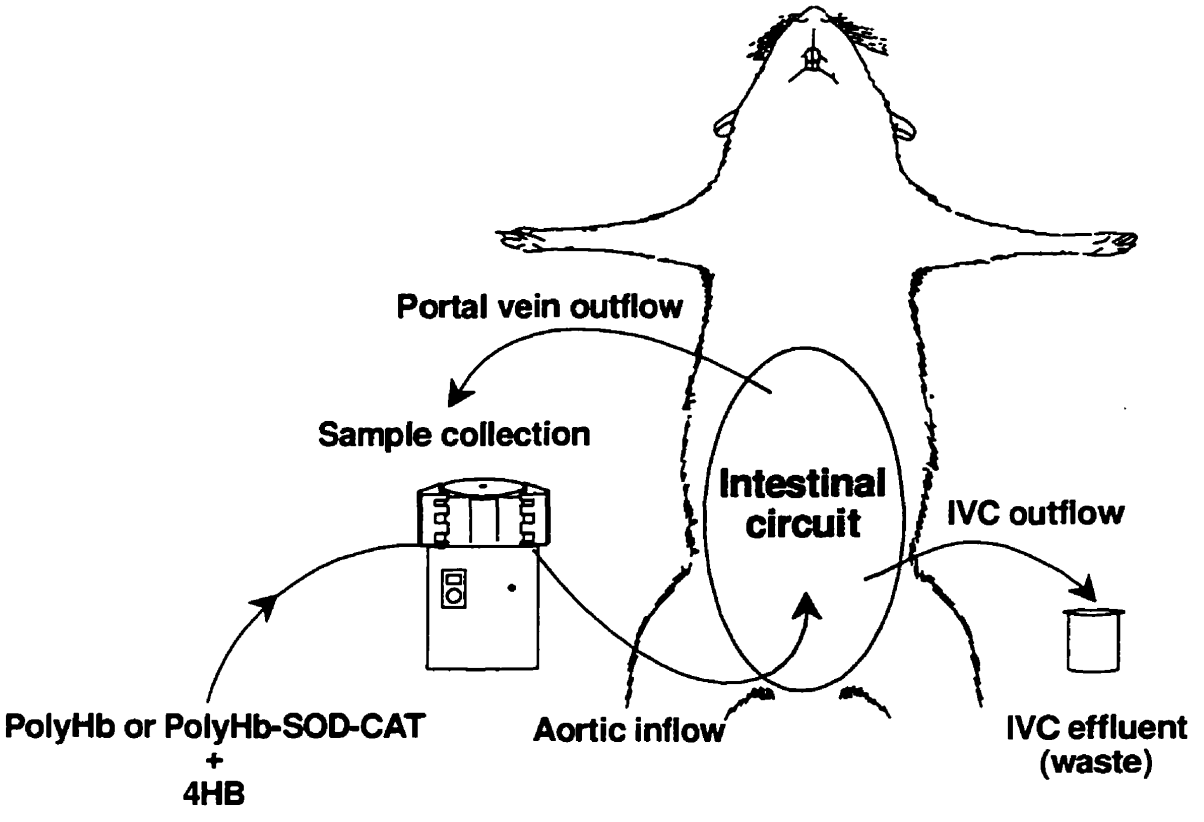

Figure 6.1 Intestinal ischemia-reperfusion model.

6.5 Results

Figure 6.2 shows the production of 3,4-DHBA during reperfusion with PolyHb and PolyHb-SOD-CAT groups. There is a significant reduction in 3,4-DHBA upon intestinal reperfusion with PolyHb-SOD-CAT versus PolyHb in this *in vivo* animal model of ischemia-reperfusion injury. The reduced formation of 3,4-DHBA suggests decreased hydroxyl radical generation. Figure 6.3 shows representative chromatograms of the two experimental and the two control groups; there is essentially ablation 3,4-DHBA generation in both controls. The greatly attenuated hydroxylation of 4HB with DMTU, a known hydroxyl radical scavenger, suggests hydroxyl radical is responsible for 3,4-DHBA production. This analytical procedure is also suitable for detecting 4HB. However, in these studies, a variety of unidentified peaks interfered with its determination. There is some evidence that 4HB can be produced by the intestine. The absence of 3,4-DHBA in the control animal treated without 4HB indicates that hydroxylation of endogenously produced 4HB can be considered negligible. The lack of 3,4-DHBA in this control also suggests that this derivative is either not produced metabolically or produced at below detectable levels.

This model provides for near complete intestinal perfusion. Ligation of the aorta and inferior vena cava proximal to the bifurcation and just above the diaphragm as well as ligation of both renal pedicles yields a closed circuit with the only inlet being the aortic cannula and the only outlet being the caval catheter. Cannulation and ligation of the portal vein further isolates the intestine. The only extraintestinal organ that is

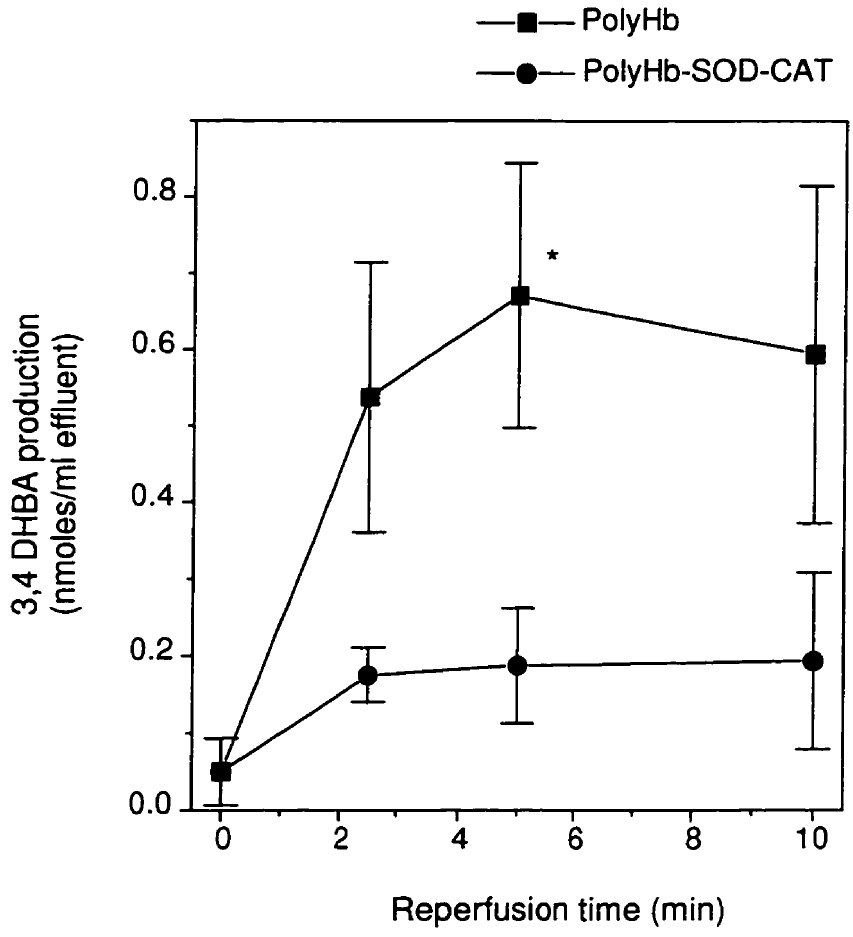

Figure 6.2 Hydroxyl radical production assessed by the measurement of 3,4 dihydroxybenzoate in ischemic intestine perfused with 5 g/dl of PolyHb (■) or 5 g/dl of PolyHb-SOD-CAT (●). Intestinal effluent samples were collected during reperfusion at the indicated times. Aliquots of these effluents were centrifuged through microconcentrators (10,000 mw cutoff). Filtrates were analyzed for 3,4 DHBA by reverse-phase HPLC using a Nucleosil column (CSC, 100 A/ODS, 5μM particle size, 25 x 0.46 cm) with Nucleosil guard column in 30mM sodium acetate-citric acid:methanol (90:10 v/v). Elution rate was 1 ml/min and the electrochemical detector potential was set at 0.85 V. Values were reported as nmole/ml effluents based on peak height determinations and standard curve obtained with authentic 3,4 DHBA. Data are presented as means ± SD. * denotes statistical significance, $p < 0.05$.

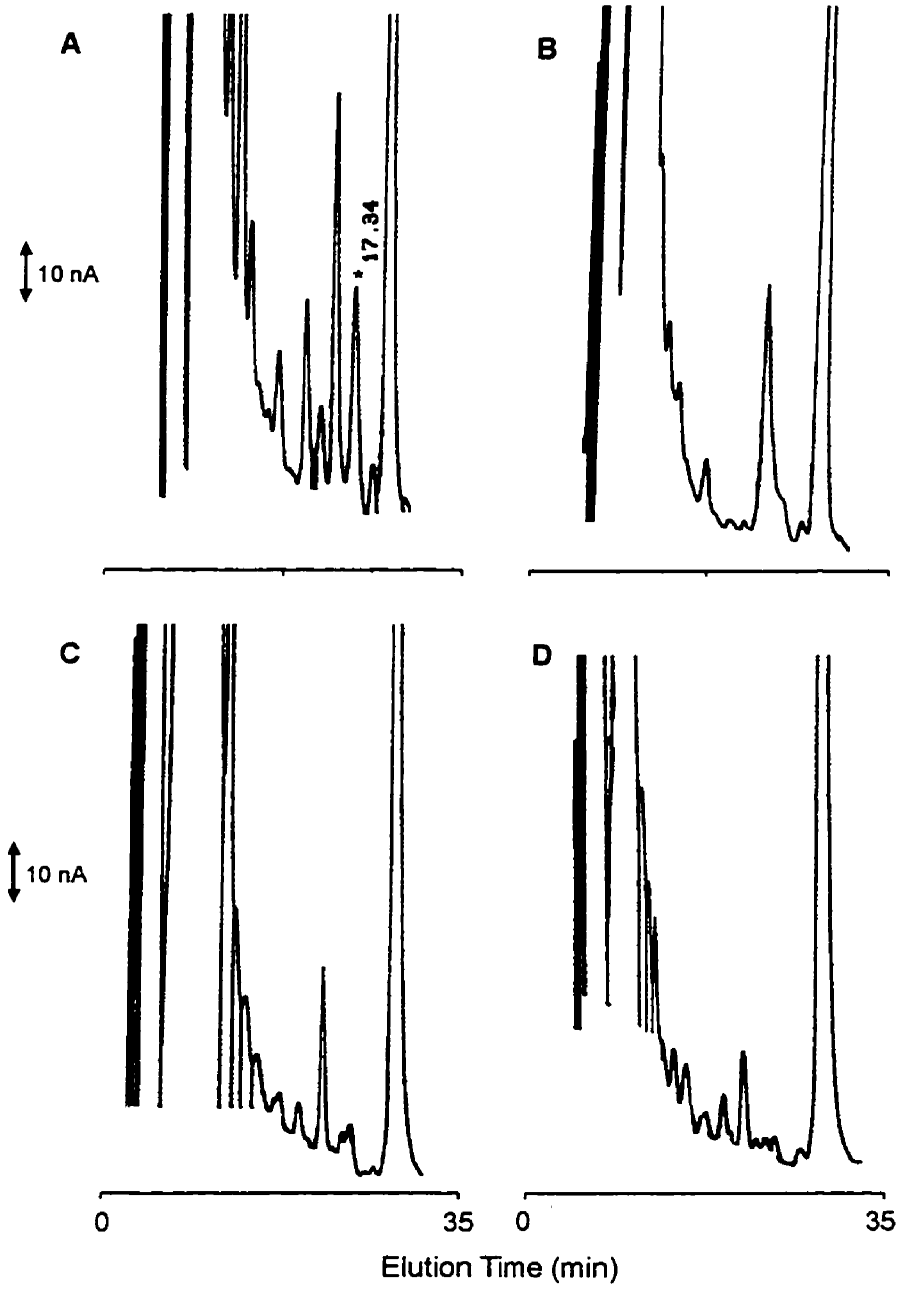

Figure 6.3 Representative chromatographs from each of the four experimental groups at 5 min of reperfusion. (A) PolyHb, (B) PolyHb-SOD-CAT, (C) PolyHb + DMTU, and (D) PolyHb alone (no 4HB). * denotes the peak representing 3,4 DHBA which elutes at 17.34 min. This peak is clearly identified in A) whereas it is nearly undetectable in all the other groups.

included in this circuit is the spleen whose arterial supply has its origin off of the celiac trunk and its venous return via the portal system through the liver [14]. Thus, the portal vein effluent samples represent mainly perfusate that perfused intestines almost exclusively.

Ischemia was taken to begin at the cessation of organ perfusion with oxygenated blood, i.e. at the time of thoracic aortic and inferior vena cava ligation. Surgical procedures performed prior to this time did not appreciably interfere with intestinal perfusion. The period of perfusion with lactated Ringer's solution prior to portal vein cannulation was necessary for technical reasons. Prior feasibility experiments revealed a high rate of portal vein thrombosis which necessitated this blood and clotting factor washout phase prior to portal vein cannulation. As well, cannulation of the portal vein *post mortem* prevents the problem of venous stasis in the intestinal veins.

6.6 Discussion

Using an *in vivo* rat model of intestinal ischemia-reperfusion injury with reversible clamping of the superior mesenteric artery (SMA), Rose et al also demonstrated an increase in hydroxyl radical generation during the early minutes of reperfusion [15]. Their assay method consisted of measuring aromatic hydroxylation products of salicylate. A marked difference, however, is that reperfusion in the Rose et al. study was conducted in the presence of polymorphonuclear cells (PMNs) of whole blood. PMNs, such as neutrophils, can significantly contribute to free radical generation through the activation of NADPH oxidase. In another SMA occlusion study, it was shown that intestinal tissue lipid peroxidation products increased upon restoration of blood flow following a 90 minute occlusion period [16]. In these studies, it was also found that intravenous infusion of PolyHb just prior to releasing occlusion did not result in further increase in lipid peroxidation products. Treatment with native superoxide dismutase and catalase significantly reduced lipid peroxidation. It is well recognized that hydroxyl radical can initiate lipid peroxidation by abstracting methylene hydrogen from polyunsaturated fatty acids. Although significant differences exist between models, the latter results casually support our data showing reduced hydroxyl radical generation with PolyHb-SOD-CAT.

Although it is clear that there is a reperfusion-mediated burst of free radical generation in models of ischemia-reperfusion, it is necessary to show that this process is at least contributory to the damage seen in reperfusion injury. Several studies have

concentrated on demonstrating that free radical generation and oxidative damage is important in the pathogenesis of the observed tissue damage. One measure of tissue injury is increased microvascular permeability. It has been shown that reperfusion with perfusates containing superoxide dismutase or copper diisopropyl salicylate (a superoxide dismutase-mimetic) is able to attenuate reperfusion-mediated increases in microvascular permeability [17]. This supports the hypothesis that the damage is at least partially mediated *via* the oxidative stress due to free radical generation. Although our results suggest attenuated hydroxyl radical generation with PolyHb-SOD-CAT *versus* PolyHb reperfusion, it is of great interest to study the actual protective benefit derived at the tissue level. Further experimentation with possibly measurements of microvascular permeability would be useful in this regard.

The attenuation in hydroxyl radical generation with PolyHb-SOD-CAT shows promise for its potential role as a protective perfusate in clinical situations of oxidative stress. An area of potential application would be the search for better organ preservation media in transplant medicine as reperfusion-mediated damage can reduce the viability of transplanted organs. Another area of the potential application of a blood substitute with antioxidant properties would be in avoiding the reperfusion injury associated with states where there is a period of no perfusion, such as occurs in cardiopulmonary bypass with circulatory arrest (CPB). Ischemia-reperfusion injury has been noted to occur after CPB [18], and reduced plasma antioxidant capacity after cardiopulmonary bypass has been demonstrated in children undergoing surgery for congenital heart disease [19].

Such potential clinical uses of PolyHb-SOD-CAT is certainly quite premature but it does provide a clinical paradigm within which to aim in further laboratory experimentation. Studies in our laboratory will continue to focus on the antioxidant properties of PolyHb-SOD-CAT with possible applications in situations of oxidative stress.

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CHAPTER 7

General Conclusions

7.1 General Conclusions

This thesis introduces a new type of modified hemoglobin oxygen carrier. The strategy I propose involves crosslinking hemoglobin and trace amounts of superoxide dismutase and catalase using the dialdehyde agent, glutaraldehyde. In the first phase of my research, I show that polymerization with glutaraldehyde has a minimal affect on the enzymatic activity of superoxide dismutase and catalase. I also show that this polymerization procedure produces a heterogeneous mixture of molecules that vary in molecular size. My circulation time studies in rats show that intermolecular polymerization increases the circulation time of hemoglobin, superoxide dismutase, and catalase. These results show the feasibility of using glutaraldehyde in this approach.

Superoxide radical and hydrogen peroxide directly or indirectly participate in the pro-oxidant mechanisms of hemoglobin. The autoxidation of hemoglobin produces superoxide radical and indirectly, via dismutation, hydrogen peroxide. These hemoglobin-derived oxidant species can themselves continue to oxidize other nearby hemoglobin molecules, and potentially lead to generation of highly reactive species, such as ferrylhemoglobin and hydroxyl radical. In the second phase of my research, I show that PolyHb-SOD-CAT prevents the formation of ferrylhemoglobin and hydroxyl radical in the presence of superoxide radical and hydrogen peroxide. In these experiments, I also show that PolyHb-SOD-CAT reduces the peroxidation of phospholipid membranes compared to PolyHb. In chapter three, I also show that the ratio of SOD:CAT affects the susceptibility of hemoglobin to oxidation in the presence

of oxidants. The results show that a low ratio of SOD:CAT is effective in preventing hemoglobin oxidation when incubated with superoxide- or hydrogen peroxide-generating enzyme systems. This observation suggests hemoglobin is more prone to oxidation by hydrogen peroxide. These results also provide useful information in determining the amount of SOD and catalase to include in formulations of PolyHb-SOD-CAT.

Alternatively, modified hemoglobin may also interact with reactive oxygen species produced in vivo. Excess production of reactive oxygen species mediates pathophysiological states, such as ischemia-reperfusion injury. The use of modified hemoglobin in these situations may further promote and exacerbate injury due to the pro-oxidant effects of hemoglobin (Figure 1.4). In the third phase of my research, I study two models of ischemia-reperfusion injury, the isolated rat hindlimb and the isolated rat intestine. In both models, reperfusion with PolyHb-SOD-CAT reduces hydroxyl radical generation compared to PolyHb. These results suggest that crosslinking SOD and catalase to hemoglobin reduces the pro-oxidant effects of hemoglobin during reperfusion of ischemic tissue. Furthermore, these results also suggest that PolyHb-SOD-CAT may be useful in preventing oxidant-mediated injury or dysfunction associated with clinical situations, such as resuscitation from hemorrhagic shock, organ preservation for transplantation, coronary angioplasty, aortic cross-clamping, and regional organ perfusion.

7.2 Claims to Originality

1. The concept of chemically crosslinking hemoglobin and the antioxidant enzymes, superoxide dismutase and catalase. (D'Agnillo F., and Chang T.M.S., U.S. Patent, Hemoglobin-Enzyme Complexes, 5,606,025, issued February 25, 1997.)

2. A novel approach in the field of modified hemoglobin oxygen carriers based on glutaraldehyde polymerization of bovine hemoglobin, bovine erythrocyte superoxide dismutase, and bovine liver catalase (PolyHb-SOD-CAT). This product consists of a wide range of molecular species which as a whole possesses beneficial antioxidant properties.

3. Crosslinking hemoglobin with SOD and catalase is feasible with glutaraldehyde in terms of allowing both antioxidants to retain enzymatic activity.

4. Superoxide dismutase and catalase activity in PolyHb-SOD-CAT prevents the formation of hydroxyl radical, ferrylhemoglobin, methemoglobin in the presence of hydrogen peroxide or superoxide radical.

5. The formulation of PolyHb-SOD-CAT should include greater activity of catalase compared to SOD. In vitro hemoglobin oxidation data suggests a ratio

of SOD/CAT of 0.01 is effective.

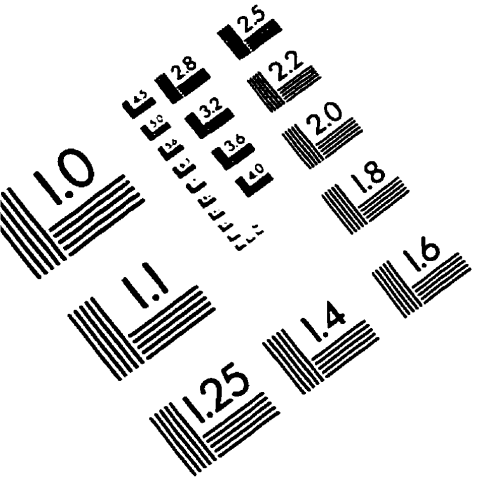
6. Reduction of hydroxyl radical formation in ischemic rat hindlimbs upon reperfusion with PolyHb-SOD-CAT as compared to PolyHb. These observations provide preliminary evidence that PolyHb-SOD-CAT is capable of reducing in vivo oxidative stress compared to PolyHb.

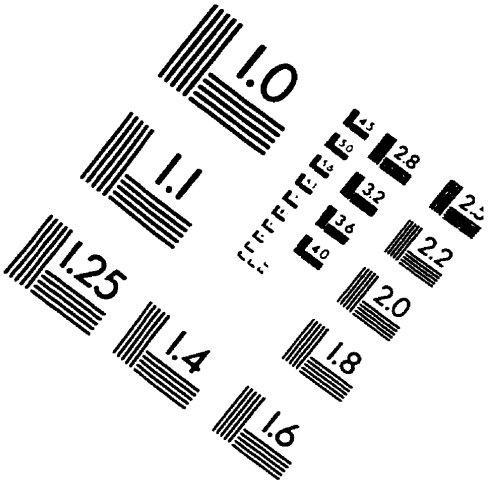
7. Reduction of hydroxyl radical formation in ischemic rat intestine upon reperfusion with PolyHb-SOD-CAT as compared to PolyHb. Further preliminary evidence suggesting that PolyHb-SOD-CAT is effective in reducing in vivo oxidative stress compared to PolyHb.

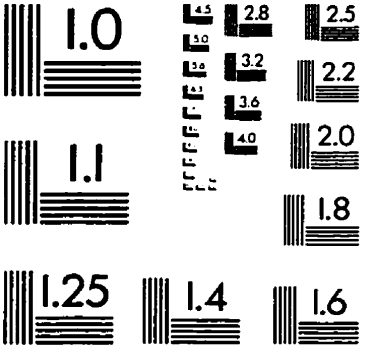
8. When crosslinked to hemoglobin, there is very significant increase in circulatory retention of superoxide dismutase and catalase activity following administration of PolyHb-SOD-CAT in Sprague Dawley rats.

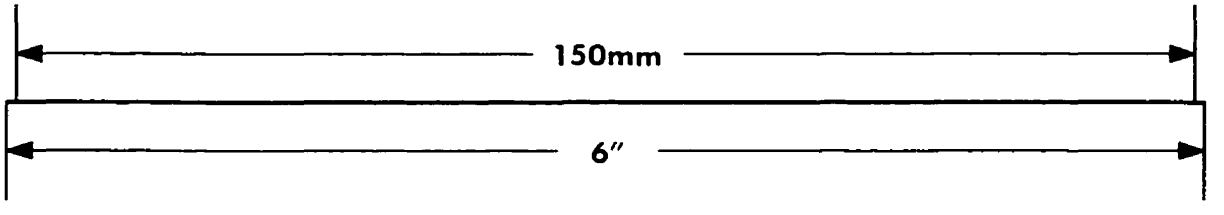
9. Based on available theoretical and experimental knowledge, these results suggest that PolyHb-SOD-CAT is a potentially safer modified hemoglobin oxygen carrier by virtue of its ability to detoxify reactive oxygen species, and its reduced propensity to promote and participate in oxidative processes.

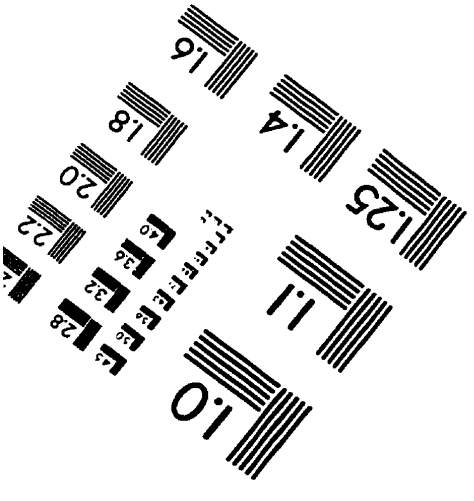
IMAGE EVALUATION
TEST TARGET (QA-3)

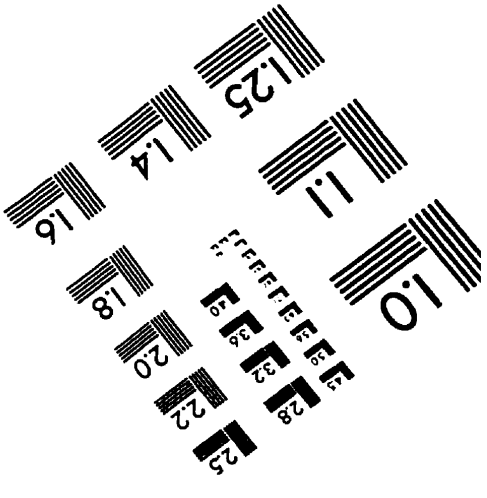












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