PHARMACOLOGICAL MECHANISMS IN THE CARDIOVASCULAR EFFECTS OF DCLHb, A HEMOGLOBIN BASED BLOOD SUBSTITUTE

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FARMACOLOGISCHE MECHANISMEN VAN DE CARDIOVASCULAIRE EFFECTEN VAN DCLHb, EEN OP HEMOGLOBIN GEBASEERDE SURROGAAT

PROEFSCHRIFT

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Dedicated to my wife Vandana
and children, Shruti and Kartike

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2. **Gulati, A.** and Rebello, S.: Diaspirin crosslinked hemoglobin (DCLHb™): involvement of adrenergic mechanisms in the pressor effect. Artif. Cells, Blood Substitutes & Immobilization Biotechnol., 22: 603-612, 1994.

3. Sharma, A.C. and **Gulati, A.**: Effect of diaspirin cross-linked hemoglobin and norepinephrine on systemic hemodynamics and regional circulation in rats. J. Lab Clin. Med. 123: 299-308, 1994.

4. **Gulati, A.** and S. Rebello: Role of adrenergic mechanisms in the pressor effect of diaspirin crosslinked hemoglobin. J. Lab Clin. Med. 124: 125-133, 1994.

5. **Gulati, A.** and Sharma, A.C.: Prazosin blocks the pressor but not the regional circulatory effects of diaspirin crosslinked hemoglobin. Life Sciences, 55: 124-130, 1994.

6. **Gulati, A.**, Sharma, A.C. and Burhop, K.: Effect of stroma-free hemoglobin and diaspirin cross-linked hemoglobin on the regional circulation and systemic hemodynamics. Life Sciences, 55: 827-837, 1994.

7. Sharma, A.C. and **Gulati, A.**: Yohimbine modulates diaspirin crosslinked hemoglobin induced systemic hemodynamics and regional circulatory effects. Critical Care Medicine, 23: 874-884, 1995.

8. **Gulati, A.**, Singh, G., Rebello, S. and Sharma, A.C.: Effect of diaspirin crosslinked and stroma reduced hemoglobin on mean arterial pressure and endothelin-1 concentration in rats. Life Sciences, 56: 1433-1442, 1995.

9. Sharma, A.C., Singh, G. and **Gulati, A.**: Role of nitric oxide mechanisms in the cardiovascular effects of diaspirin crosslinked hemoglobin in anesthetized rats. Amer. J. Physiol., 269: H1379-H1388, 1995.

10. **Gulati, A.**, Singh, R., Chung, S.M. and Sen A.P.: Role of endothelin converting enzyme in the systemic hemodynamics and regional circulatory effects of proendothelin-1 [1-38] and diaspirin crosslinked hemoglobin in rats. J. Lab. Clin. Med., 126: 559-570, 1995.

11. **Gulati, A.**: Development of artificial blood substitutes. Drugs: News and Views, 3: 102-104, 1995.

12. **Gulati, A.**, Sharma, A.C. and Singh, G.: Role of endothelin mechanism in the cardiovascular effects of diaspirin crosslinked and stroma free hemoglobin. Critical Care Medicine, 24: 137-147, 1996.

13. **Gulati, A.**, Sen A.P., Sharma, A.C. and Singh, G.: Role of endothelin and nitric oxide mechanisms in the resuscitative effect of diaspirin crosslinked hemoglobin, a hemoglobin based blood substitute, following hemorrhage in rats. Amer. J. Physiol., 1996 (under revision).

14. **Gulati, A.**, Sen A.P. and Singh, R.: Effect of diaspirin crosslinked hemoglobin, a hemoglobin-based blood substitute, on the regional blood circulation in severely hemorrhaged rats. Amer. J. Physiol., 1996.

15. Sen A.P.,Dong, Y. and **Gulati, A.**: Effect of diaspirin crosslinked hemoglobin, a hemoglobin based blood substitute, on systemic and regional blood circulation in pregnant rats. Artif. Cells, Blood Substitutes & Immobilization Biotechnol., 1996.

16. Dong, Y., Sen, A.P. and **Gulati, A.**: Modulation of the cardiovascular effects of diaspirin crosslinked hemoglobin, a hemoglobin based blood substitute, by N^G-nitro-L-arginine methyl ester in hemorrhaged rats. Critical Care Medicine 1996.

Table of contents

Part 4: Role of nitric oxide mechanism in the cardiovascular effects of diaspirin crosslinked haemoglobin

Part 5: Role of endothelin mechanism in the cardiovascular effects of diaspirin crosslinked haemoglobin

Part 1

Introduction

Chapter 1

1.1 Introduction and Background

The search for a clinically useful blood substitute has been stimulated by the inherent limitations of the homologous blood transfusion system, particularly its sufficiency, safety and costs. Blood has been described as the "most complicated fluid in animals" (Winslow, 1992). An attempt to formulate a blood substitute is misguided because blood is composed of a complex mixture of fluids, cells, salts, proteins and numerous other molecules having various functions and characteristics. The hemorrhage or loss of blood foremost results in a hypovolemic state and ultimately leads to depletion of oxygen delivering capability (Dracker, 1995).

Biologically significant hemorrhage may be defined as a blood loss sufficient to impair oxygen transport (Shoemaker *et al.*, 1973; Bassin *et al.*, 1971; Hauser and Shoemaker, 1982). The major physiological effect of hemorrhage is anemia and hypovolemia leading to drastic alterations in blood flow to the vital organ systems (Prough *et al.*, 1991). The persistent hypoperfusion to organ systems is responsible for ultimate organ failure even after reperfusion (Knaus *et al.*, 1985). The infusion of large volume of Ringer's lactate in the resuscitation of hemorrhaged patients is associated with several disadvantages. In addition, problems associated with blood transfusion necessitate the development of an alternative resuscitative solution which can be administered in small volumes and is effective in combating the compromised hemodynamics. Haemoglobin-based blood substitutes (oxygen carriers) have been proposed to be effective in the treatment of hemorrhagic shock.

The major categories of blood substitutes include the (1) perfluorocarbon solutions and (2) haemoglobin based compounds.

1.2 Problems associated with use of perfluorocarbons as blood substitutes

The perfluorocarbons are water insoluble, halogenated hydrocarbon compounds that are liquid at body temperature and are excellent solvents for oxygen and other gases. The advantages of perfluorocarbon products are they are synthetic in nature and the problem of transmission of human disease is nearly eliminated. These products are chemically inert and are unlikely to undergo biodegradation or generation of toxic or antigenic products. The manufacturing process is very simple and commercial quantities can be produced at relatively low costs. However, in contrast to haemoglobin, which binds oxygen cooperatively and is nearly saturated with oxygen when breathing at room air, perfluorocarbons have a high solubility for oxygen. The higher the concentration of oxygen inspired, the more the oxygen will be dissolved. Therefore, significant clinical benefit requires the use of supplemental oxygen and a very high FIO_2 is needed to carry

sufficient oxygen. The particle size of perfluorocarbons is also critical and if it is too large, capillary plugging may occur and if the particle is too small, an increase in blood viscosity may occur. Following intravenous administration the perfluorocarbon droplets are gradually removed from the circulation by the reticulo-endothelial system and a large dose of perfluorocarbon may result in the enlargement of the organs of the reticulo-endothelial system.

1.3 Problems associated with use of haemoglobin based blood substitutes

Early attempts to prepare haemoglobin-based blood substitutes involved hypotonic lysis of human red blood cells followed by crude separation of the soluble haemoglobin from the insoluble cell membranes. These solutions produced significant nephrotoxicity and intravascular coagulation. During 1960's techniques were developed to eliminate 99% of the red cell stroma, but these stroma-free haemoglobin solutions still produced severe adverse effects. There are two major limitations in the use of stroma-free haemoglobin as resuscitative solution. The first is that the haemoglobin tetramer dissociates into dimers. This dissociation is extremely rapid and when haemoglobin is administered it is rapidly excreted in urine. The second limitation is that in the absence of its normal allosteric regulator 2,3 DPG the affinity of haemoglobin for oxygen is very high and this does not allow unloading of oxygen to the tissues.

A safe and effective oxygen carrying haemoglobin-based blood substitute could be life saving for trauma victims and has become a major goal of many research groups within industry, academic centers, and the military. Oxygen carrying haemoglobin-based blood substitutes have numerous advantages over conventional blood transfusions. They can be produced in large volume, stored for prolonged periods (about one year or more), administered rapidly and without need for cross-typing, and can be made free of viruses and other infectious agents (Estep *et al.*, 1989a; Estep *et al.*, 1989b). A number of haemoglobin-based blood substitutes have passed significant phase I safety hurdles and are now in phase II patient testing (Dracker, 1995; Chang, 1995).

1.4 Current technology: An overview

Intrinsic interest in the structure and function of haemoglobin has resulted in a large number of chemical modifications. Free haemoglobin has been cross linked, conjugated, polymerized or encapsulated to prevent its dissociation into dimers. Intramolecular bifunctional cross-linking reagent bis(N-maleimidomethyl) ether, increases the half-life of haemoglobin by 4 folds but also increased the oxygen affinity. Cross-linking has also been done to dextran or hydroxyethyl

starch. Intermolecular cross-linking has been performed using glutaraldehyde to produce oligomers larger than haemoglobin tetramers.

Several types of haemoglobin-based blood substitutes have been developed and are in different phases of clinical trials. Studies have demonstrated the efficacy of haemoglobin-based blood substitutes in resuscitation following hemorrhage in animals. Crystalline haemoglobin solution to treat severe hypovolemia and anemia in separate canine models and found it to be highly effective, suggesting that it acts as a plasma expander to resuscitate intravascular volume and also supplements oxygen carrying capacity in anemia (Hauser and Shoemaker, 1982). Microencapsulated and conjugated haemoglobin were found to be effective in hemorrhagic shock (Usuba *et al.*, 1992; Agishi *et al.*, 1988; Iwashita *et al.*, 1988; Rabinovici *et al.*, 1992). Crosslinked haemoglobin solutions have also been demonstrated to be effective in the resuscitation of hemorrhagic shock (Keipert and Chang, 1985; Hess *et al.*, 1992; Malcolm *et al.*, 1992; Ning *et al.*, 1992a; Rabinovici *et al.*, 1989).

Isovolemic exchange transfusion with 25% of total estimated blood volume in anesthetized dogs using 9 g/dl of SFHb produced a transient rise in the mean arterial blood pressure with a simultaneous increase in the blood flow to vital organs. However, on the renal circulation SFHb produced decrease in the glomerular filtration rate (Ning *et al.*, 1992b). 50% exchange transfusion following liposomal encapsulated haemoglobin though maintains mean arterial blood pressure but causes increase in total peripheral resistance and decrease in cardiac output (Rabinovici *et al.*, 1992). Total exchange transfusion with liposomal encapsulated haemoglobin has been found to sustain blood pressure and cardiac output but reduced systemic vascular resistance (Miller *et al.*, 1988). An ultrapurified, polymerized, bovine haemoglobin solution has been shown to improve the cerebral blood flow when administered to hemorrhaged rats. Stroma free polymerized bovine haemoglobin has been found to be effective in restoring the systemic hemodynamic parameters in hemorrhaged dogs. Another study using polymerized ultrapurified bovine haemoglobin has shown that it is effective in restoring the cardiovascular parameters following ovarian hemorrhage in a miniature horse. A liposome encapsulated haemoglobin preparation consisting of lyophilized powder for use as "instant blood" has also been described. The use of haemoglobin-based blood substitutes will introduce a new approach in critical care medicine of not only improving perfusion but delivering oxygen to tissues.

Diaspirin crosslinked haemoglobin (DCLHb) is a modified haemoglobin solution derived from human erythrocytes. It is prepared by cross-linking haemoglobin the α-subunits, within the haemoglobin tetramer, by means of a reaction with the diaspirin compound, bis(3,5-dibromosalicyl) fumarate. It is purified by heat pasteurization to inactivate any contaminating

viruses and precipitate undesirable proteins. It possesses biochemical stability and exhibits greater intravascular retention than unmodified haemoglobin. It is slowly degraded in the blood stream and has less accumulation in the tissues, and it is catabolized to low molecular weight compounds which are eliminated through the urine and feces. DCLHb does not require cross-matching or typing prior to administration, is less viscous than whole blood, and may be better able to carry oxygen through narrowed vessels to ischemic tissues due to the smaller size of the haemoglobin molecule relative to the erythrocytes. DCLHb is devoid of white cells and other blood components which are known to contribute to ischemic tissue injury by releasing cytotoxic products. DCLHb has been found not to elicit any inflammatory reactions in sheep and monkeys. It does not interfere with the coagulation cascade, or the reticular endothelial system. Studies have shown that DCLHb produces significant increases in mean arterial pressure (MAP) in normal anaesthetized and conscious rats.

1.5 Role of endogenous vasoactive substances in the cardiovascular effects of haemoglobin-based blood substitutes

In addition to binding oxygen, haemoglobin is known to have a very high affinity for nitric oxide (NO) (Gibaldi, 1993). NO which is bound by haemoglobin may be converted to nitrate through interaction with the heme group (Wennmalm *et al.*, 1992). The NO system is often found to be involved in the regulation of regional blood circulation in normal animals. L-NAME, a NO synthase (NOS) inhibitor, blocked the increases in the rat cortical blood flow due to stimulation of cerebrovascular parasympathetic system (Morita-Tsuzuki *et al.*, 1993) and decreased cerebral blood flow in goats (Fernandez *et al.*, 1993). Both L-NAME and another NOS inhibitor, N^G-Nitro-L-arginine, produced constriction of coronary arterioles in dogs (Jones *et al.*, 1993). L-NAME decreased renal blood flow and increased renal vascular resistance in rats (Sigmon *et al.*, 1993), attenuated the vasodilation of muscular arterioles in response to acetylcholine in hamsters (Hester *et al.*, 1993), and decreased cerebral blood flow and increased cerebral vascular resistance in dogs (Saito *et al.*, 1994). Endogenous NO mediates most of its actions through cGMP (Thiemermann, 1994; Moncada and Higgs, 1993). It is possible that following hemorrhage an increase in the release of NO occurs leading to an increase in cGMP concentration in the blood plasma. The removal of NO and decrease in cGMP is likely to contribute toward restoring the vascular tone and responsiveness.

In physiological states, a delicate balance exists between vasoconstrictor substances (ET) and vasodilator substances (EDRF/NO) to maintain vascular tone (Rubanyi, 1991; Zingarelli *et al.*, 1992). At the level of the endothelial cell, pro-ET (bigET) is cleaved to ET, the biologically active product (Yanagisawa *et al.*, 1988; Gulati and Srimal, 1992). This conversion of bigET to

ET is inhibited by phosphoramidon, a metallo-protease inhibitor (Matsumura *et al.*, 1992). To counter balance the constrictor properties of ET, EDRF/NO is released (Rubanyi, 1991).

1.6 Role of endogenous vasoactive substances in hemorrhagic shock

The endothelium-derived vasoactive agents which regulate the vascular tone have been implicated in the pathophysiology of hemorrhagic shock (Henrich, 1991). Vascular endothelial cells have been reported to generate NO, a potent vasodilator (Palmer *et al.*, 1988). Severe hemorrhagic hypotension causes hyporeactivity of the cardiovascular system to catecholamines. This is mediated by an increase in NOS activity and release of NO (Thiemermann *et al.*, 1993). In a study, resuscitation with L-NAME (10 mg/kg, i.v.) increased mean arterial pressure in urethane anaesthetized rats hemorrhaged for 20 min with 50% survival rate at 120 min (Zingarelli *et al.*, 1992). In urethane anaesthetized rats hemorrhaged for 15-20 min to a mean arterial pressure of 60 mmHg, L-NAME (0.3-30 mg/kg, i.v.) dose-dependently increased mean arterial pressure (Chyu *et al.*, 1992). L-NAME (44 mg/kg, i.v.) pretreatment increased mean arterial pressure but decreased HR in conscious hemorrhaged rabbits (Koch *et al.*, 1995). NOS inhibitor, N^G-methyl-L-arginine (30 mg/kg, i.v.) has been shown to restore mean arterial pressure in pentobarbital anaesthetized rats hemorrhaged for 2 mins (Klabunde *et al.*, 1993), infusion of another NOS inhibitor N^G-monomethyl-L-arginine (1.2 mg/kg/min) increased mean arterial pressure in hemorrhaged rats (Lieberthal *et al.*, 1991). Endogenous NO mediates most of its actions through cGMP (Thiemermann, 1994; Moncada and Higgs, 1993) and it appears that an increase in cGMP levels in hemorrhage is deleterious to the survival of the animal. The studies available in literature so far have focused on the improvement in mean arterial pressure following hemorrhage. However, in order to determine efficacy it is essential to evaluate the improvement in base deficit, oxygen consumption, systemic hemodynamics, regional blood circulation and survival time after resuscitation. Thus, the role of NO inhibitors in resuscitation following hemorrhage still remains unclear.

The role of ET in the regulation of cardiovascular system has been suggested (Gulati, 1995; Gulati *et al.*, 1995; Sharma and Gulati, 1995; Stojilkovic and Catt, 1992; Hay *et al.*, 1993). Though ET mechanisms have been shown to participate in the pathogenesis of several cardiovascular disorders including myocardial ischemia, congestive heart failure, hypertension and in a variety of shock syndromes, its role in hemorrhagic shock is not clear. It was demonstrated that the elevation in plasma endothelin levels occurs during central hypovolemia in humans (Matzen *et al.*, 1992). The elevation of plasma ET-1 concentration has been reported following hemorrhage in rats (Vemulapalli *et al.*, 1994; Zimmerman *et al.*, 1994) and dogs (Chang *et al.*, 1993). In a normal situation the circulating ET-1 level is low and it is released

more toward the abluminal side of the endothelial cells (Gulati, 1995). ET-1 and ET-3 can release NO in rat and rabbit isolated perfused preparations (Warner *et al.*, 1989). It has been suggested that this release might be through ET receptors.

1.7 Aims of the present study

The following were the aims of the present study:

1. To study the cardiovascular effects of DCLHb, a haemoglobin based blood substitute.

2. To compare the cardiovascular effects of DCLHb with known pressor agents and with unmodified haemoglobin solution.

3. To determine whether adrenergic mechanism is involved in the cardiovascular effects of DCLHb.

4. To determine whether nitric oxide mechanism is involved in the cardiovascular effects of DCLHb.

5. To determine whether endothelin mechanism is involved in the cardiovascular effects of DCLHb.

6. To determine the efficacy of DCLHb in an animal model of severe hemorrhage.

7. To determine the pharmacological mechanism involved in the resuscitative effect of DCLHb in hemorrhaged rats.

Part 2

Cardiovascular effects of diaspirin crosslinked haemoglobin

Chapter 2

Regional circulatory and systemic hemodynamic effects of diaspirin cross-linked haemoglobin in the rat

Summary

Diaspirin cross-linked haemoglobin (DCLHb™) (Baxter Healthcare Corporation) is a promising resuscitative fluid. The effect of DCLHb (400 mg/kg, iv), on regional circulation and systemic hemodynamics was studied in male Sprague-Dawley rats using a radioactive microsphere technique. Systemic hemodynamics, distribution of cardiac output, regional blood flow and vascular resistance were determined before (baseline) and 15, 30 and 60 min after the administration of DCLHb. Infusion of an equal volume of saline did not produce any significant change in systemic hemodynamics or regional circulation. DCLHb produced an increase (79%) in the mean blood pressure which lasted for more than 60 min. Heart rate, cardiac output and stroke volume were not significantly affected, while total peripheral resistance was increased after the administration of DCLHb. DCLHb produced significant increases in blood flow to the heart, gastrointestinal tract (GIT), portal system and skin. The blood flow to kidney, brain and musculoskeletal system was not significantly affected by DCLHb. The vascular resistance was not altered in the heart, brain, GIT, portal system, kidney or skin, but there was a marked increase in the vascular resistance in the musculoskeletal system. There was a significant increase in the percentage of cardiac output to visceral organs like heart, GIT and portal system, while a marked decrease in the percent cardiac output to musculoskeletal system was observed with DCLHb. It is concluded that the blood flow to most of the organs is either increased or is not affected by DCLHb.

2.1 Introduction

There has been tremendous progress in the development of haemoglobin solutions as resuscitative solutions. Several investigators have stressed a number of advantages of haemoglobin solutions over other resuscitation solutions (Bonhard, 1975; Kaplan and Murphy, 1975; Moss *et al.*, 1976; DeVenuto and Zegna, 1978). Diaspirin cross-linked haemoglobin (DCLHb) is a blood substitute derived from the human erythrocytes. DCLHb is produced by cross-linking molecular haemoglobin between the α-subunits by means of a reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The cross-linking of the α subunits affords the haemoglobin a favorable oxygen dissociation curve (Snyder *et al.*, 1987; Vandegriff *et al.*, 1989). The manufacturing process also includes heat pasteurization of the solution (Estep *et al.*, 1989b; Estep *et al.*, 1989a). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986).

DCLHb has been found to be an effective resuscitative fluid following hemorrhage (Przybelski *et al.*, 1990). It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions are not affected significantly (Hess *et al.*, 1989). DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters (Przybelski *et al.*, 1991) in a rodent hemorrhage model. DCLHb has also been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion (Cole *et al.*, 1992) in rats.

In normal adult rats DCLHb has been found to increase the mean arterial blood pressure (Hamilton *et al.*, 1992), while several other haemoglobin solutions have been reported to produce a rapid and sustained increase in mean arterial pressure (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). Whereas purified stroma-free haemoglobin solutions have been reported to produce vasoconstrictor activity in a variety of experimental models (Wellum *et al.*, 1980; Vogel *et al.*, 1986; Gilroy *et al.*, 1988). Yamakawa et al. reported that stroma-free haemoglobin solution produced marked vasodilatation of coronary blood vessels, when administered to the rat (Yamakawa *et al.*, 1990). However, the effect of DCLHb on the regional blood circulation has not been studied. The present study was conducted to determine the effect of DCLHb on regional blood flow and systemic hemodynamics in the rat.

2.2 Materials and Methods

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 300-350 g were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683. The carotid artery of the right side was exposed and a PE 50 cannula guided through the common carotid artery to the left ventricle. The femoral artery of the right side was cannulated and the cannula guided to the abdominal aorta and connected to a withdrawal pump (Harvard Model 22).

At each measurement, a suspension of approximately 200,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA) in 0.2 ml saline were injected into the left ventricle after thoroughly mixing and flushed with 0.4 ml saline over a 15 sec period. In order to calculate

the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the catheter inserted in the abdominal aorta via the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: brain, kidneys, heart, gastrointestinal tract (stomach, small intestine, caecum, large intestine, mesentry and pancreas), liver, spleen, skin and the rest of the body consisting of muscles and bones. The radioactivity in the microspheres injected, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output (2) stroke volume (3) total peripheral resistance (4) regional blood flow and (5) regional vascular resistance. The data was calculated using the computer programs (Saxena *et al.*, 1980). Infusion of saline did not affect the systemic hemodynamics or regional circulation. The effect of DCLHb (400 mg/kg, iv) on systemic hemodynamics and regional circulation was studied. The dose of DCLHb was selected on the basis of the earlier studies conducted in several laboratories (Hamilton *et al.*, 1992; Gulati and Rebello, 1994).

All data are presented as the mean values ± 1 SEM. Data were analyzed by analysis of variance followed by Duncan's or Scheffe's S test. A level of $P < 0.05$ was considered significant.

2.3 Results

Effect of DCLHb on the systemic hemodynamics

DCLHb (400 mg/kg, i.v.) produced a significant [$F(3, 20) = 11.28$; $p = 0.008$) increase in the mean arterial blood pressure 15, 30 and 60 min after administration. The heart rate, cardiac output and stroke volume were not affected after DCLHb administration. DCLHb significantly increased the total peripheral resistance at 15 and 60 min [$F (3,20) = 2.72$; $p = 0.04$] (Table 1).

Effect of DCLHb on the regional blood flow

DCLHb (400 mg/kg, iv) significantly increased [$F (3,20) = 4.09$; $p = 0.026$] the blood flow to the heart, at 15, 30 and 60 min after infusion (Fig. 1). Blood flow was significantly increased to the GIT [$F (3,20) = 3.62$; $p = 0.022$] and portal system [$F (3,20) = 3.54$; $p = 0.03$] 15 and 30 min after DCLHb infusion and skin [$F (3,20) = 2.71$; $p = 0.03$] 30 min after the administration of DCLHb (Fig. 2). Blood flow to the brain, kidney and musculoskeletal system was not significantly affected by DCLHb (Fig. 2).

Effect of DCLHb on the regional vascular resistance

DCLHb did not affect the vascular resistance in the heart (Fig. 1) brain, GIT, portal system, kidney and skin (Fig. 3). The vascular resistance was found to be significantly increased in the musculoskeletal system [$F(3,20) = 5.57$; $p = 0.01$] at 15, 30 and 60 min after the administration of DCLHb (Fig. 3).

Effect of DCLHb on the distribution of cardiac output

The percent cardiac output was significantly increased to the heart [$F(3,20) = 10.44$; $p = 0.01$] at 15, 30 and 60 min after DCLHb infusion and the portal system [$F(3,20) = 4.05$; $p = 0.02$] and GIT [$F(3,20) = 2.72$; $p = 0.04$] 15 min after infusion. On the other hand, the percent cardiac output to the musculoskeletal system was found to be significantly decreased [$F(3,20) = 5.39$; $p = 0.01$] 15, 30 and 60 min after DCLHb administration. The percent cardiac output to the brain, kidney and skin was not altered by DCLHb administration (Fig. 4).

Table 1: Effect of DCLHb (400 mg/kg, iv) on the systemic hemodynamics in rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)	386 ± 10	397 ± 10	398.3 ± 11	395 ± 10
Mean B.P.(mmHg)	84 ± 6	150 ± 9*	144 ± 11*	125 ± 9*
Cardiac output (ml/min)	82 ± 10	107 ± 7	119 ± 20	86 ± 6
Stroke Volume (ml)	0.2 ± 0.02	0.3 ± 0.02	0.3 ± 0.06	0.2 ± 0.02
TPR (MBP/CO)	1002 ± 82	1493 ± 149*	1347 ± 207	1570 ± 110*

*$p < 0.05$ as compared to control; $N = 6$.

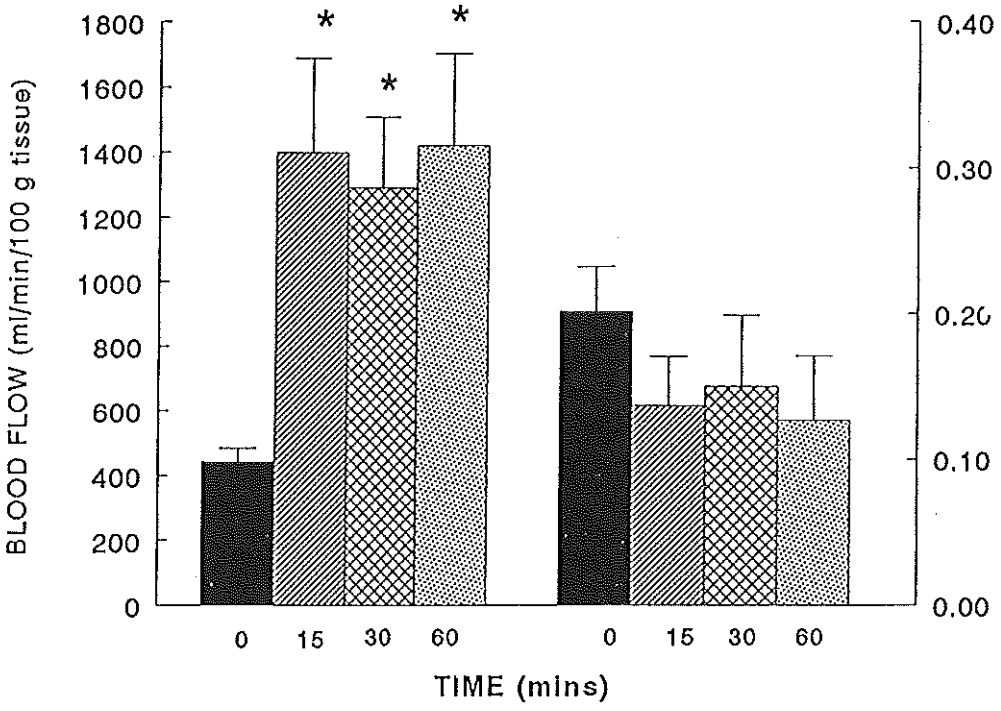

Fig. 1 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow to the heart (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) before (0) and at 15 min, 30 min and 60 min after its administration to the rats. Asterisks indicate significant difference as compared to control.

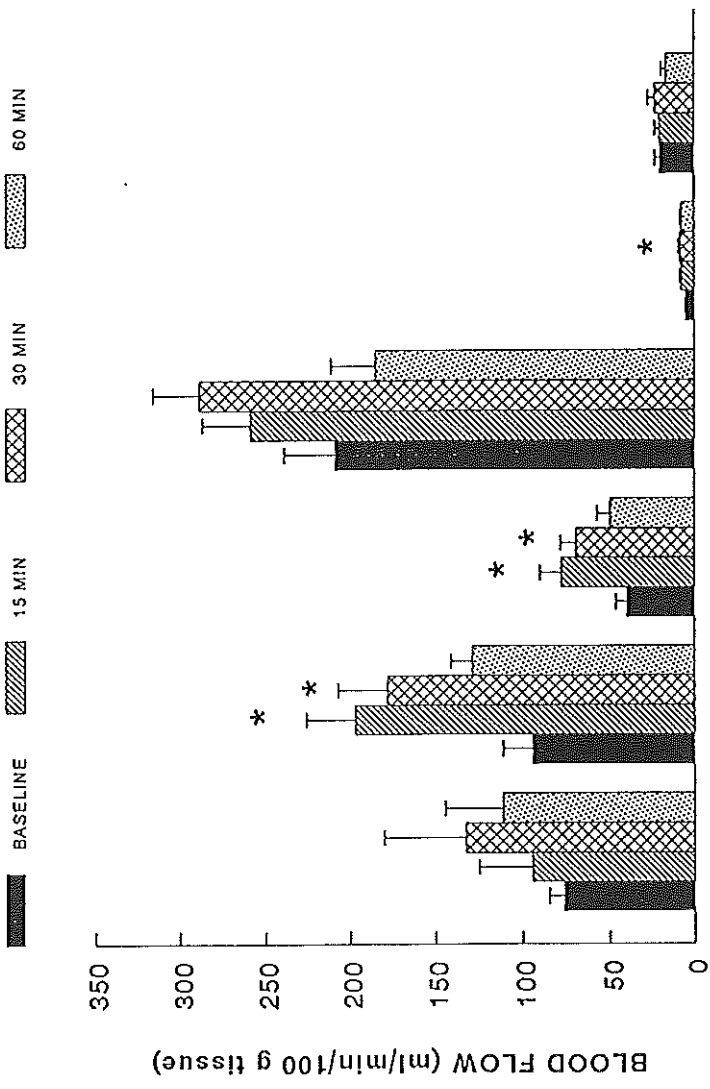

Fig. 2 The effect of DCLHb (400 mg/kg, i.v.) on the regional blood flow (ml/min/100 g tissue) before (0) and at 15 min, 30 min and 60 min after its administration to the rats. Asterisks indicate significant difference as compared to control.

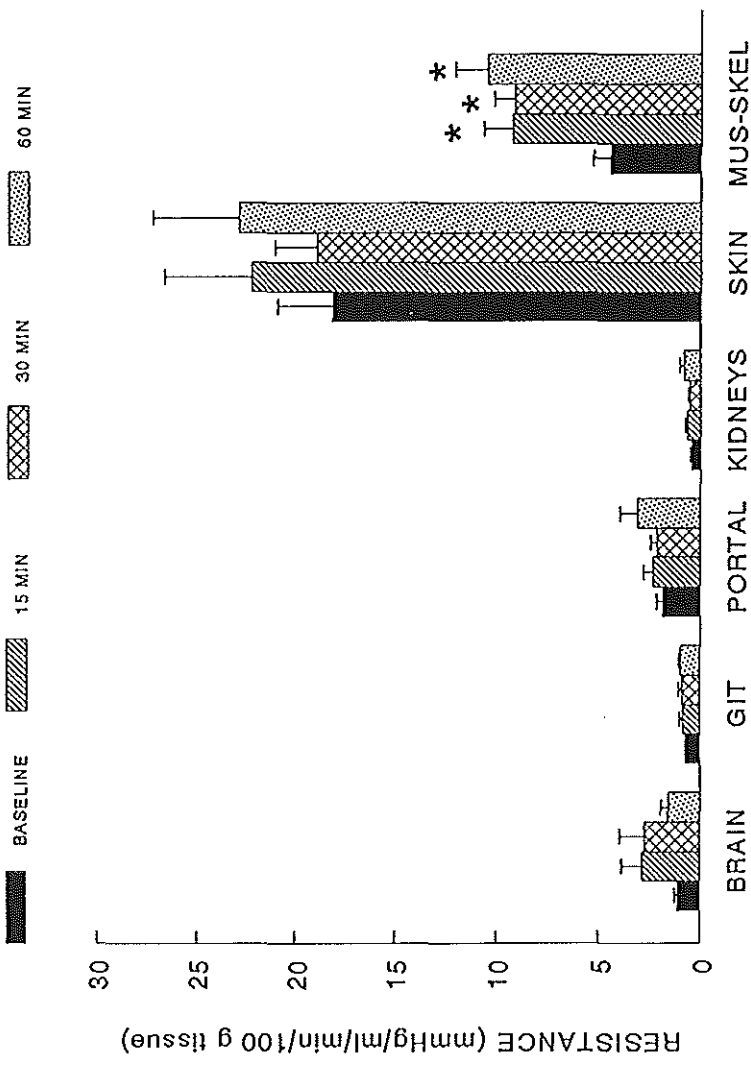

Fig. 3 The effect of DCLHb (400 mg/kg, i.v.) on the regional vascular resistance (mmHg/ml/min/100 g tissue) before (0) and at 15 min, 30 min and 60 min after its administration in rats. Asterisks indicate significant difference as compared to control.

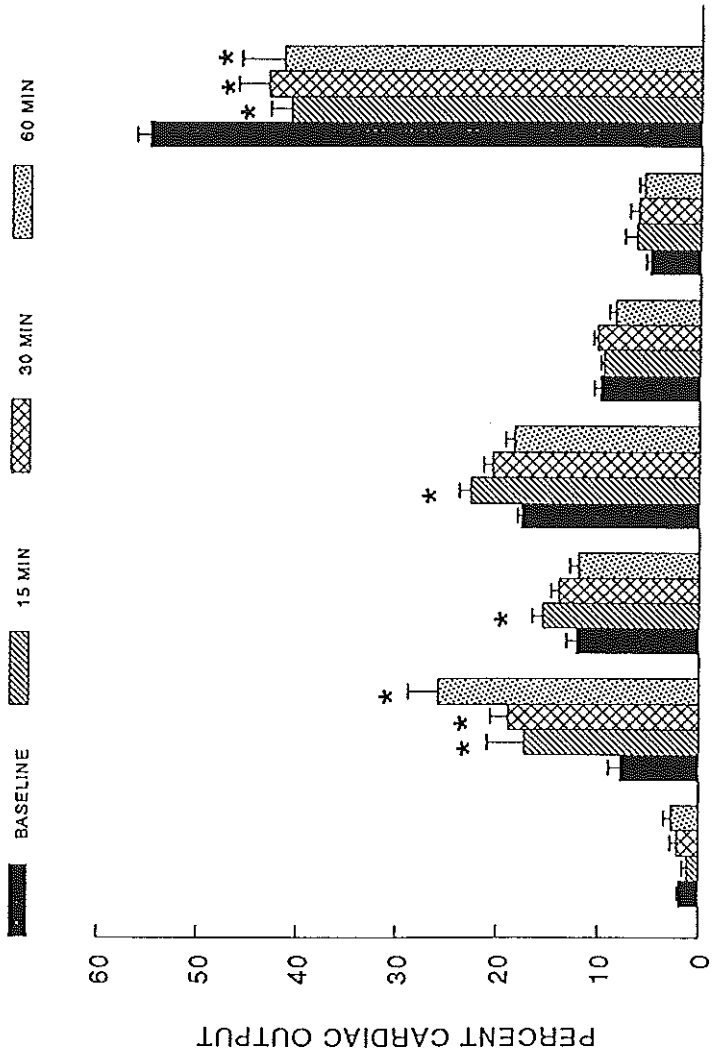

Fig. 4 The effect of DCLHb (400 mg/kg, i.v.) on the percent cardiac output to various tissues before (0) and at 15 min, 30 min and 60 min after its administration to the rats. Asterisks indicate significant difference as compared to control.

2.4 Discussion

Stroma-free haemoglobin solutions have been reported to produce a pressor effect (Przybelski *et al.*, 1991; Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989; Kida *et al.*, 1991). DCLHb has also been demonstrated to produce a pressor response in rats (Hamilton *et al.*, 1992; Kida *et al.*, 1991). In the present study DCLHb was found to produce a marked increase in the mean arterial pressure and total peripheral resistance in rats. Cardiac output, stroke volume and heart rate were not altered. The blood flow to the heart, GIT, portal system and skin increased while it was not altered in brain, kidney or the musculoskeletal system. The vascular resistance was not affected in most of the organs except in the musculoskeletal system in which a marked increase was observed. It appears that the blood flow to most of the visceral organs is increased, probably due to the redistribution of blood from the musculoskeletal system. This was confirmed by determining the percent cardiac output to various organs systems. It was found that the percent cardiac output to heart, GIT and portal system is increased while it is significantly decreased to the musculoskeletal system. Since the major portion (55%) of the cardiac output goes to the musculoskeletal system, a small decrease in percent cardiac output to the musculoskeletal system could lead to a significant increase in the blood flow to other organs. It is concluded that DCLHb increases the blood flow to several visceral organs due to the redistribution of blood flow from the musculoskeletal system.

Haemoglobin solutions or red cell lysates have been shown to possess vasoconstrictor actions on various types of arteries in different animals (Gilroy *et al.*, 1988; Yamakawa *et al.*, 1990; Biro, 1982). However, it is not clear whether it is the haemoglobin molecule or some other associated factors responsible for the vasoconstrictor effect. The vasoconstriction induced by haemoglobin has been attributed to the haemoglobin molecule or a very closely associated contaminant (Vogel *et al.*, 1986; Gilroy *et al.*, 1988).

Studies have demonstrated that chemical modifications of haemoglobin by conversion to met-haemoglobin (Vogel *et al.*, 1986) or by glutaraldehyde cross-linking (Vogel *et al.*, 1986; Vogel *et al.*, 1988) can significantly reduce the constrictor activity of haemoglobin. The vasoconstrictor activity is reduced in haemoglobin solutions purified by affinity chromatography or preparative HPLC (Vogel *et al.*, 1987) but not by membrane dialysis or ultrafiltration (Vogel *et al.*, 1986). Removal of stromal fraction has been demonstrated to diminish vasoconstrictor action in isolated rabbit heart (Biessels *et al.*, 1992). MacDonald et al. found that cross-linked haemoglobin solution has less vasoconstrictor activity as compared with unmodified haemoglobin on coronary blood vessels (Macdonald *et al.*, 1990). Lieberthal et al. also found that cross-linked haemoglobin solution did not while unmodified haemoglobin solution significantly increased the perfusion pressure and the resistance of the coronary blood vessels

(Lieberthal *et al.*, 1989). These studies suggested that besides chemical modification, the stromal fraction could be contributing towards vasoconstrictor activity.

DCLHb is a chemically modified (intramolecular cross-linking of α subunits) and highly purified (including heat pasteurization) haemoglobin solution. The present study using an in vivo model shows that the blood flow to most of the organs is either increased or is not affected by administration of DCLHb.

2.5 Acknowledgments

The authors are grateful to Dr. P.R. Saxena, Erasmus University, Rotterdam for providing the computer program to calculate the cardiovascular parameters.

Chapter 3

Effect of stroma-free haemoglobin and diaspirin cross-linked haemoglobin on the regional circulation and systemic hemodynamics

Summary

The effects of unmodified stroma-free haemoglobin (SFHb) and diaspirin cross-linked haemoglobin (DCLHb) on the regional blood circulation and systemic hemodynamics were studied in rats using a radioactive microsphere technique. SFHb and DCLHb increased mean arterial blood pressure without affecting heart rate. SFHb produced a 24.9 % decrease in the cardiac output while DCLHb produced an 44.8 % increase in the cardiac output. Stroke volume was decreased (-27.3 %) by SFHb and increased (+36.4 %) by DCLHb. Total peripheral resistance increased with both SFHb and DCLHb. DCLHb increased blood flow to the heart, spleen, stomach, small intestine and skin, and had no effect on blood flow to the brain, kidneys, liver, mesentery, pancreas, caecum, large intestine and musculo-skeletal system. In contrast, in animals infused with SFHb, blood flow decreased to the kidneys, liver and spleen, increased to the heart, small intestine and skin, and had no effect to the brain, caecum, large intestine and musculo-skeletal system. DCLHb had no effect on vascular resistance in any organ except for an increase in the musculo-skeletal system. In contrast, SFHb increased vascular resistance in the kidneys, liver, spleen, skin, mesentery and pancreas, and had no effect on vascular resistance in the musculo-skeletal system, brain, heart, stomach, small intestine, caecum and large intestine. SFHb had no effect on distribution of cardiac output to the brain, gastrointestinal tract (GIT), kidneys, skin, musculo-skeletal and portal system, while DCLHb significantly decreased the percent cardiac output to the musculo-skeletal system. DCLHb did not affect the distribution of cardiac output to the brain, GIT, kidneys, skin and portal system. SFHb and DCLHb increased the percent cardiac output to the heart. It is concluded that similar concentrations and doses of DCLHb and SFHb produce different effects on the regional blood circulation and systemic hemodynamics.

3.1 Introduction

The use of modified haemoglobin solutions as blood substitutes has been extensively investigated and haemoglobin solutions are being developed as promising resuscitative solutions. Diaspirin cross-linked haemoglobin (DCLHb) is a blood substitute derived from the human erythrocytes. It is prepared by cross-linking molecular haemoglobin between the α-subunits by means of a reaction with the diaspirin compound, bis(3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process also includes heat pasteurization of the solution (Estep *et al.*, 1989b; Estep *et al.*, 1989a) to inactivate any contaminating viruses and precipitate undesired proteins. DCLHb has been found to be effective in the resuscitation of rats following

hemorrhagic shock (Przybelski *et al.*, 1990), even at volumes one-half that of whole blood (Przybelski *et al.*, 1991). DCLHb, after partial or complete exchange transfusion in swine, was found to maintain cardiac and renal functions (Hess *et al.*, 1989).

Haemoglobin solutions have been reported to produce a rapid and sustained increase in mean arterial pressure (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). DCLHb has been shown to produce a dose-dependent increase in blood pressure which was self-limiting and a dose of 400 mg/kg resulted in a near maximal pressor response (Hamilton *et al.*, 1992). Studies have reported that haemoglobin solutions produce vasoconstrictor activity which could be due to the activity of haemoglobin molecule itself or due to the presence of other factors in the haemoglobin solution (Gilroy *et al.*, 1988). Purified SFHb solutions produce vasoconstrictor activity in a variety of experimental models (Gilroy *et al.*, 1988; Vogel *et al.*, 1986). Haemoglobin has been found to be a vasoconstrictor for the cerebral blood vessels both *in vivo* (Sonobe and Suzuki, 1978; Kajikawa *et al.*, 1979) and *in vitro* (Wellum *et al.*, 1980). A coronary vasoconstrictor effect of human SFHb was identified using an isolated rabbit heart preparation (Vogel *et al.*, 1986). In contrast, other studies have been reported that demonstrate a vasodilator effect of haemoglobin solutions. For example, when SFHb was administered to the rat there was a vasodilation of coronary blood vessels, which was not observed with a pyridoxalated haemoglobin polyoxyethylene conjugate (Yamakawa *et al.*, 1990). Myocardial blood flow, determined by using radioactive microspheres, was increased by SFHb solution in dogs (Biro, 1982). In rabbit hearts an increase in the coronary perfusion pressure was observed with HPLC purified haemoglobin and by unmodified SFHb solution, while a diaspirinated haemoglobin solution produced a minimal effect on coronary perfusion pressure (Lieberthal *et al.*, 1989). Recently, it has been reported that unmodified human SFHb solution produces an increase in the blood flow to the heart, brain, liver, gut and kidneys of dog (Ning *et al.*, 1992).

It seems likely that haemoglobin solutions have different actions in various vascular beds, i.e. producing vasoconstriction in some beds while producing vasodilatation in others. It also seems that different haemoglobin preparations produce different effects, and the model chosen, i.e. *in vivo* vs *in vitro*, could affect the results. The present study was conducted with the aim of determining the effect of SFHb on the regional circulation and systemic hemodynamics in rats using an *in vivo* model since most of the above studies were conducted on isolated tissues or organs. Another aim of the study was to compare the effect of unmodified SFHb solution with a modified and chemically stabilized haemoglobin solution, DCLHb, on the regional circulation and systemic hemodynamics.

3.2 Materials and Methods

Drugs

Diaspirin cross-linked haemoglobin (DCLHb) was prepared (Azari *et al.*, 1993) and provided by Baxter Healthcare Corp., Round Lake, IL. Stroma-free haemoglobin (SFHb) was prepared at Baxter Healthcare Corp., Round Lake, IL by a method similar to that described earlier (Zager and Gamelin, 1989). Briefly, outdated human RBCs were separated by centrifugation and lysed by adding 2 vol of distilled water per 1 vol of RBCs followed by two freeze thawing procedures. The lysed cells were centrifuged for 15 min at 26,000 g. The supernatant containing SFHb was collected and used for subsequent testing. The physicochemical properties of SFHb and DCLHb are shown in table 1.

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 340-390 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant, and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683. The right carotid artery was exposed and a PE 50 cannula guided through the common carotid artery to the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P 23 DC pressure transducer. When the cannula reached the ventricle the diastolic pressure dropped to zero. The right femoral artery was also cannulated and the catheter was connected to a withdrawal pump (Harvard Model 22).

Determination of systemic hemodynamics and regional circulation

At each measurement, a suspension of approximately 200,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA) and thoroughly mixed with 0.2 ml saline were injected into the left ventricle. The catheter was flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all the tissues and organs were dissected out, weighed and

placed in vials. The following tissues were studied: lungs, heart, liver, stomach, small intestine, caecum, large intestine, mesentery and pancreas, spleen, left kidney, right kidney, left cerebral hemisphere, right cerebral hemisphere, midbrain, cerebellum, brain stem, skin and rest of the body consisting of muscles and bones. The radioactivity in the standards, the blood samples, and the tissue samples, were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output, (2) stroke volume, (3) total peripheral resistance, (4) regional blood flow and (5) regional vascular resistance. The data were calculated using the programs described earlier (Saxena *et al.*, 1980). Infusion of an equal volume of saline (4 ml/kg, iv) did not affect the systemic hemodynamics and regional circulation. The effects of SFHb (400 mg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on the systemic hemodynamics and regional circulation were studied. The doses were selected on the basis of studies conducted previously (Hamilton *et al.*, 1992; Gulati and Rebello, 1994; Sharma and Gulati, 1994) which demonstrated that the infusion of this dose of DCLHb resulted in a near maximal pressor response.

Statistics

All data are presented as the mean values ± 1 SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Duncan's test. A level of $P < 0.05$ was considered significant.

3.3 Results

Effect of SFHb and DCLHb on systemic hemodynamics

SFHb produced a significant ($F(3,16) = 3.73$; $P = 0.02$) increase in mean arterial blood pressure 15 and 30 min after infusion. DCLHb, on the other hand, produced a more marked and prolonged increase ($F(3,24) = 16.26$; $P = 0.0001$) in blood pressure at 15, 30 and 60 min after infusion. Neither SFHb nor DCLHb produced a significant effect on heart rate. SFHb produced a slight decrease in the cardiac output (-23.2 %) which was not statistically significant, while DCLHb produced a significant (45.3 %) increase in the cardiac output. Stroke volume was slightly decreased by the infusion of SFHb (-27.3 %), while DCLHb produced a 36.4 % increase in the stroke volume. Both SFHb and DCLHb significantly increased the total peripheral resistance (table 2).

Table 1 Physicochemical properties of unmodified (SFHb) and modified (DCLHb) haemoglobin solutions.

	SFHb	DCLHb
Total haemoglobin (g/dl)	10.4 g/dl	10.0 g/dl
Pyrogenicity (LAL) EU/ml	< 0.06	< 0.125
Methaemoglobin %	12.8 %	4.1 %
Sodium (mEq/l)	114	141
Chloride (mEq/l)	137	112
Sterility	Sterile	Sterile
Phospholipids	>50 ppm	0.2 ppm
Cross-linking	-------	99.9 %
p50	-------	31 mmHg

Effect of SFHb and DCLHb on the regional brain circulation

The blood flow to the cerebral hemispheres, diencephalon, cerebellum and brain stem was not altered by infusion of DCLHb or SFHb, although there was a tendency towards an increase in the regional brain blood flow by SFHb but not by DCLHb (fig. 1). The vascular resistance was not altered in any region of the brain (fig. 1).

Effect of SFHb and DCLHb on the coronary circulation

SFHb produced a significant ($F(3,16) = 5.08$; $P = 0.02$) increase in the blood flow to the heart 15 and 30 min after infusion. DCLHb also produced a significant ($F(3,24) = 3.51$; $P = 0.03$) increase in the blood flow 15, 30 and 60 min after infusion (fig. 2). The vascular resistance in the heart did not change significantly following infusion of either DCLHb or SFHb (fig. 2).

Effect of SFHb and DCLHb on the renal circulation

DCLHb did not affect blood flow or vascular resistance in left or right kidney after infusion (fig. 3). SFHb, on the other hand, produced a significant decrease in blood flow to the left ($F(3,16) = 2.89$; $P = 0.03$) and right ($F(3,16) = 6.61$; $P = 0.004$) kidneys at 60 min. The vascular resistance was also significantly ($F(3,16) = 8.18$; $P = 0.002$) increased by SFHb 30 and 60 min after infusion (fig. 3).

Effect of SFHb and DCLHb on the portal circulation

SFHb significantly decreased blood flow to the liver ($F(3,16) = 2.36$; $P = 0.03$) and spleen ($F(3,16) = 3.41$; $P = 0.04$) 60 min after infusion (fig. 4). After infusion of SFHb, vascular resistance was found to be significantly ($F(3,16) = 2.93$; $P = 0.03$) increased 30 min after administration in the liver and 30 and 60 min after administration in the spleen ($F(3,16) = 4.68$; $P = 0.02$). DCLHb, on the other hand, did not affect the blood flow or vascular resistance in the liver, but significantly ($F(3,24) = 2.89$; $P = 0.03$) increased the blood flow to the spleen 15 min after infusion. The blood flow to the spleen returned to normal by 60 min after infusion. The vascular resistance was not affected following the infusion of DCLHb in the liver and the spleen (fig. 4). The blood flow and vascular resistance in the mesentery and pancreas were not affected by infusion of either SFHb or DCLHb.

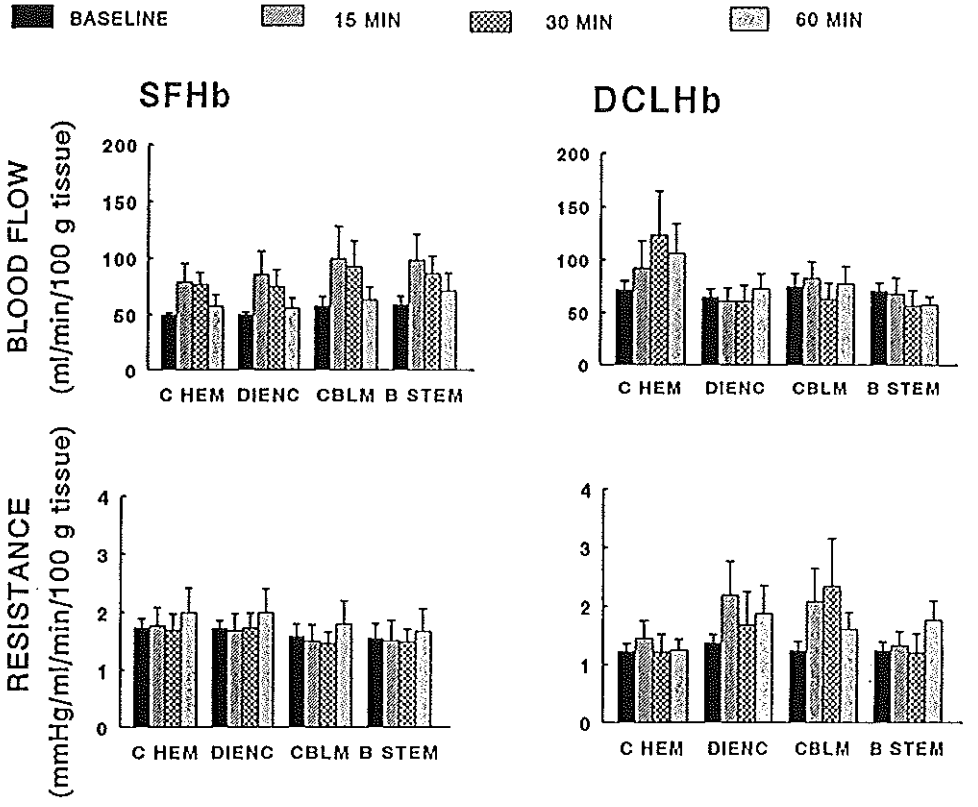

Fig. 1 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on blood flow and vascular resistance in various regions of the brain (C HEM = cerebral hemispheres; DIENC = diencephalon; CBLM = cerebellum; B STEM = brain stem). The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats.

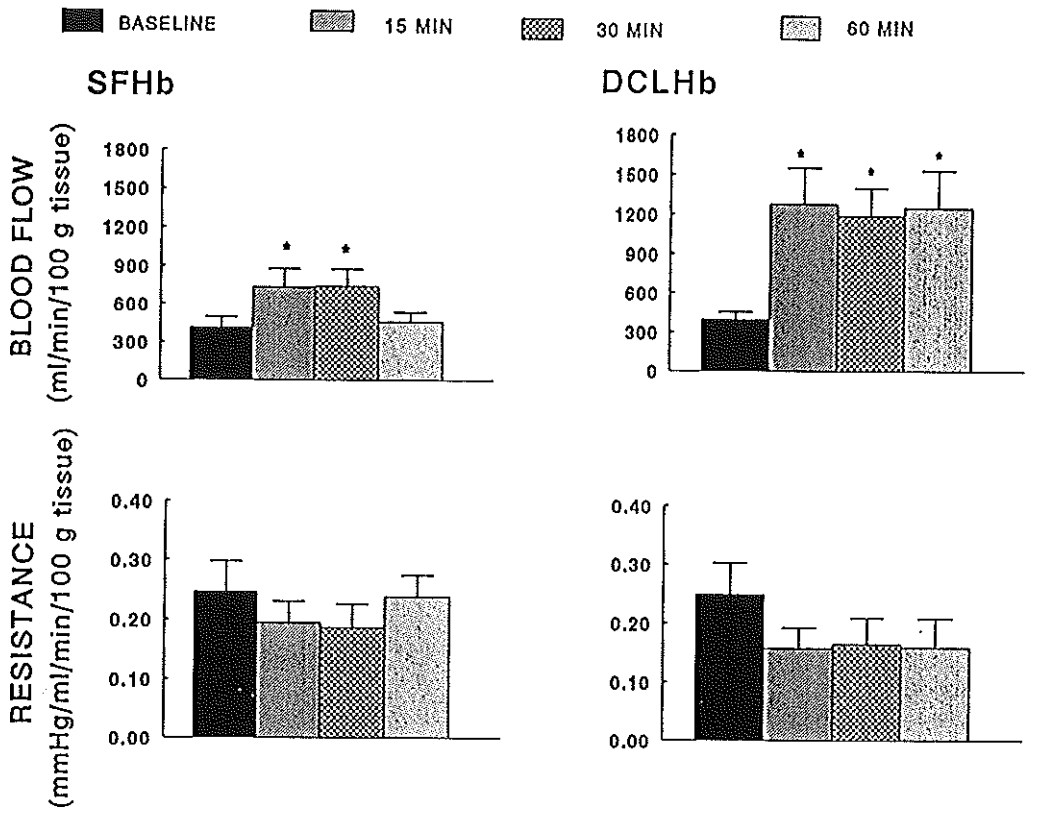

Fig. 2 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on blood flow and vascular resistance in the heart. The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats. Asterisks indicate significant difference as compared to baseline.

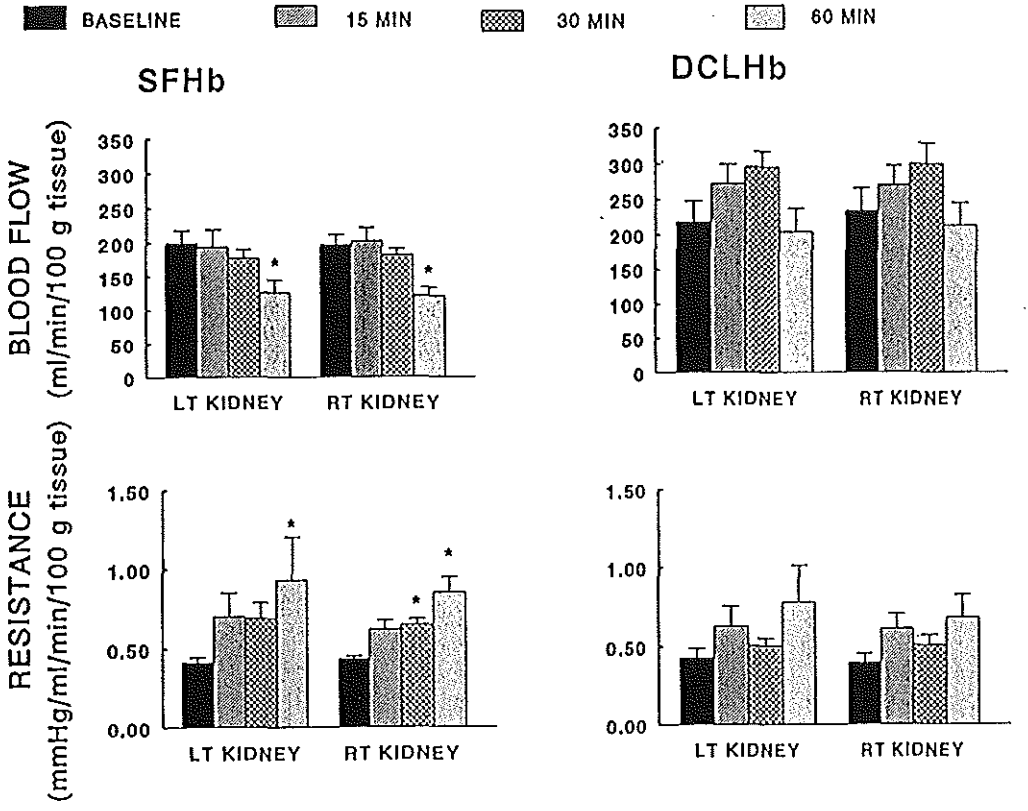

Fig. 3 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on blood flow and vascular resistance in the left and right kidneys. The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats. Asterisks indicate significant difference as compared to baseline.

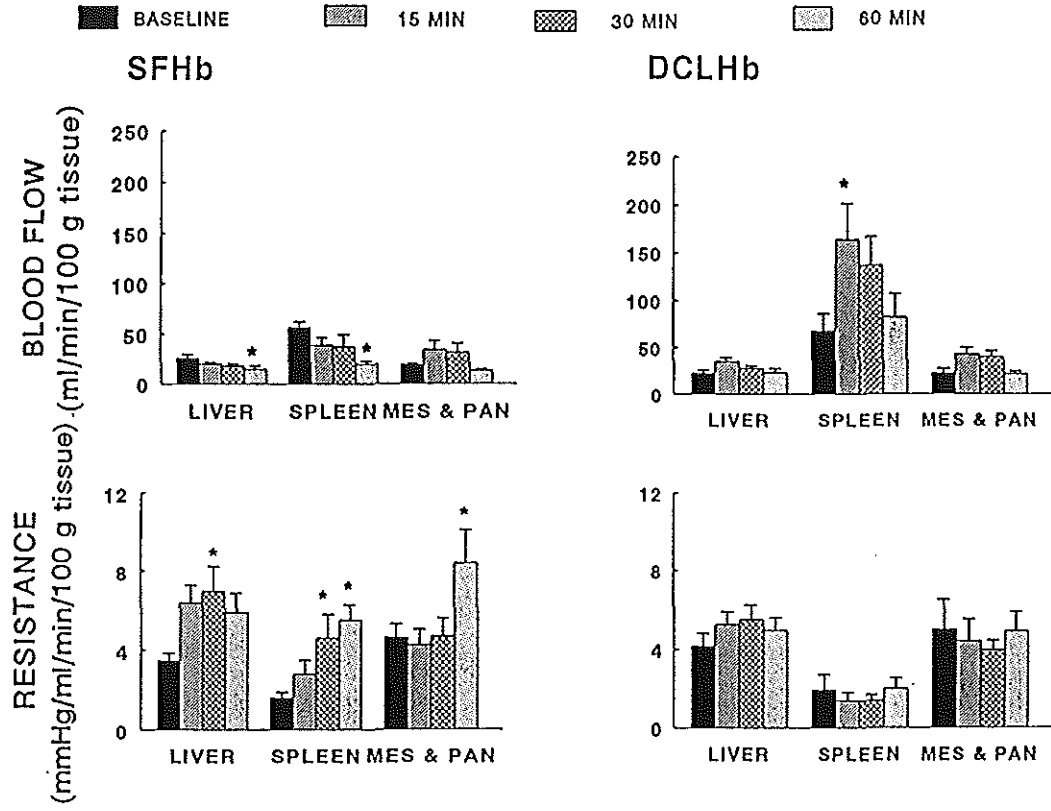

Fig. 4 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on blood flow and vascular resistance in the liver, spleen, mesentery and pancreas. The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats. Asterisks indicate significant difference as compared to baseline.

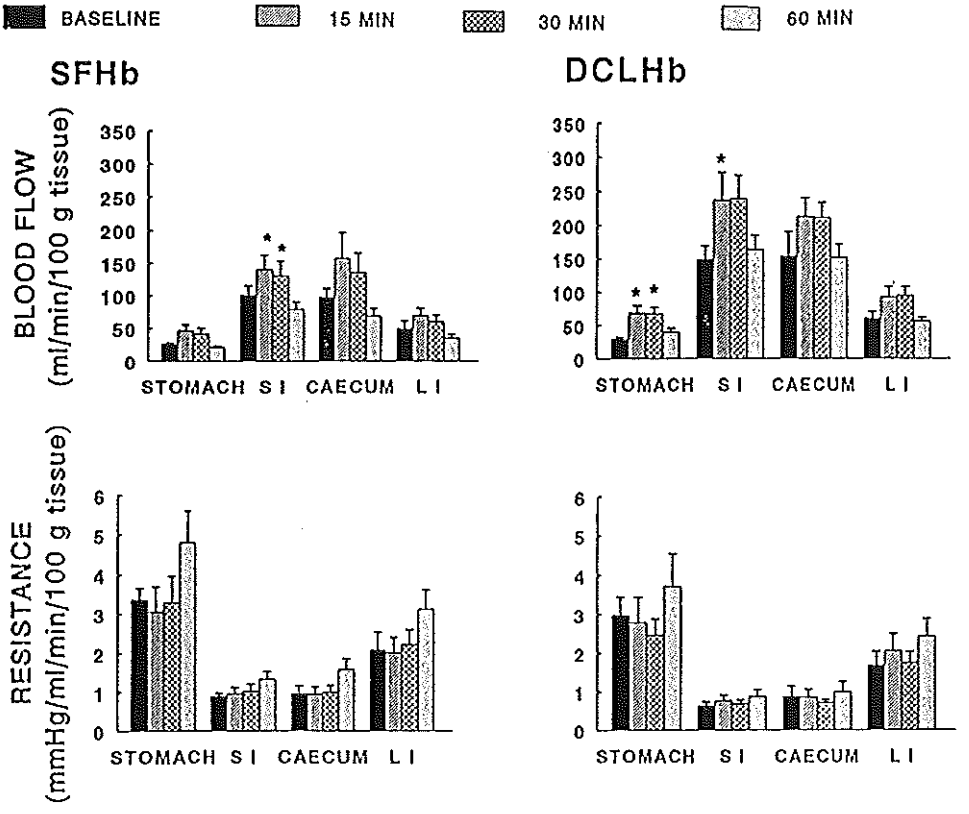

Fig. 5 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on blood flow and vascular resistance in the stomach, small intestine (SI), caecum and large intestine (LI). The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats. Asterisks indicate significant difference as compared to baseline.

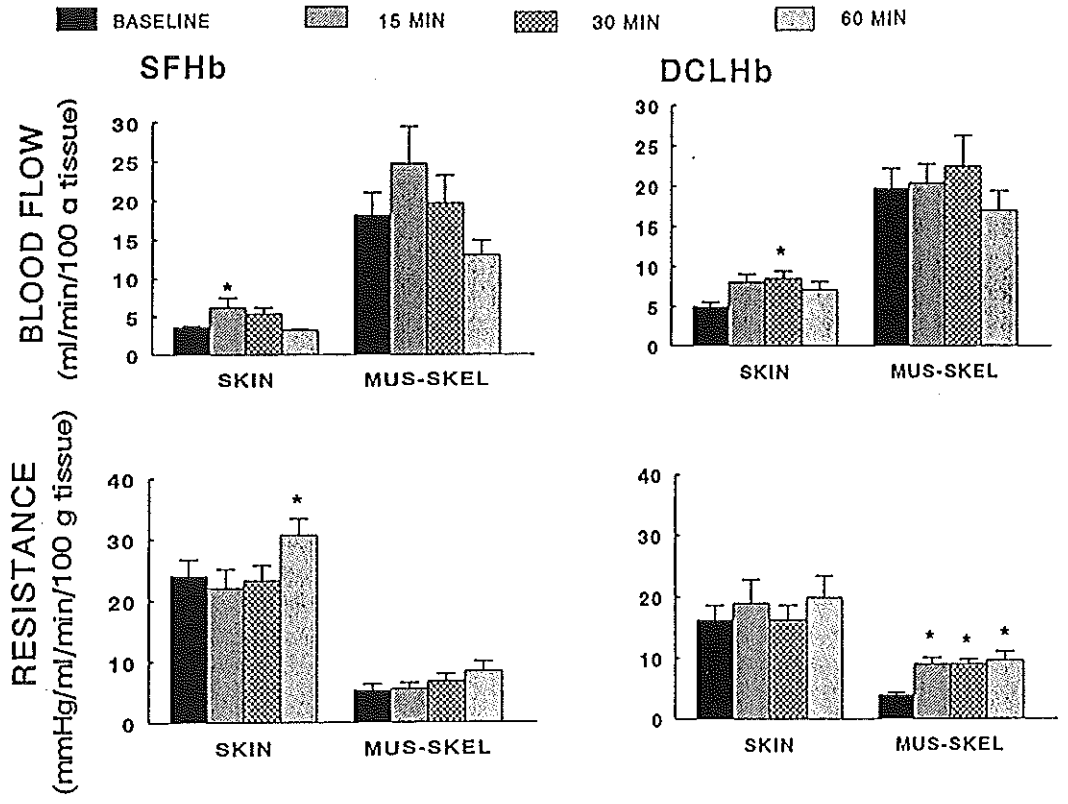

Fig. 6 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on blood flow and vascular resistance in the skin and musculo-skeletal system. The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats. Asterisks indicate significant difference as compared to baseline.

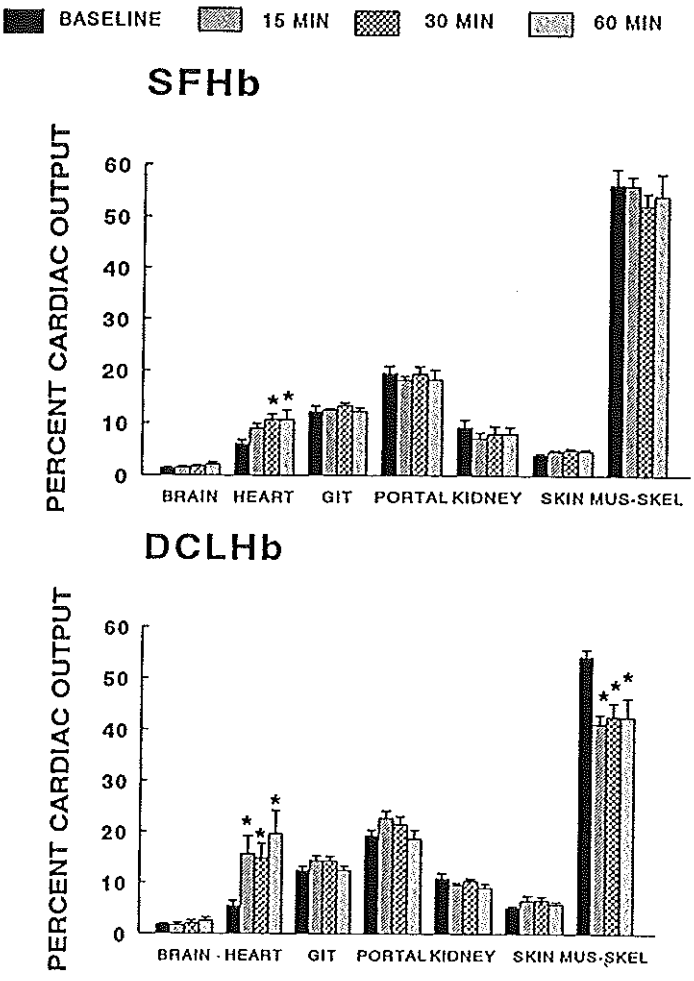

Fig. 7 The effect of DCLHb (400 mg/kg, i.v.; N=7) and SFHb (400 mg/kg, i.v.; N=5) on the percent distribution of cardiac output to the brain, heart, gastrointestinal tract, portal system, kidneys, skin and musculo-skeletal system. The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after the administration of either DCLHb or SFHb to rats. Asterisks indicate significant difference as compared to baseline.

Table 2 Effect of intravenous infusion of SFHb (400 mg/kg) and DCLHb (400 mg/kg) on systemic hemodynamics parameters.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)				
SFHb	318 ± 29	380 ± 19	380 ± 18	340 ± 36
DCLHb	377 ± 13	400 ± 9	402 ± 10	396 ± 8
Blood pressure (mmHg)				
SFHb	84.5 ± 5.2	123.0 ± 9.7*	118.3 ± 10.4*	99.8 ± 10.4
DCLHb	82.1 ± 5.6	150.5 ± 7.3*	143.2 ± 9.5*	124.0 ± 7.6*
Cardiac output (ml/min)				
SFHb	87.6 ± 11.4	96.0 ± 7.7	87.7 ± 16.0	65.8 ± 6.4
DCLHb	82.6 ± 8.0	111.5 ± 7.4	119.6 ± 16.5*	90.4 ± 7.1
Stroke volume (ml)				
SFHb	0.30 ± 0.07	0.33 ± 0.08	0.29 ± 0.06	0.21 ± 0.4
DCLHb	0.22 ± 0.02	0.28 ± 0.02	0.30 ± 0.05	0.23 ± 0.02
Total peripheral resistance (mmHg/l/min)				
SFHb	1035 ± 149	1271 ± 137	1282 ± 229	1574 ± 203*
DCLHb	1028 ± 79	1399 ± 137	1315 ± 178	1419 ± 129*

*Indicates significant ($P < 0.05$) difference as compared to control.

Effect of SFHb and DCLHb on the GIT circulation

Although, not statistically significant, SFHb increased (82 ± 35 %) blood flow to the stomach 15 min after infusion. DCLHb significantly ($F(3,24) = 5.41$; $P = 0.005$) increased blood flow to the stomach 15 and 30 min after infusion. The vascular resistance in the stomach was not affected by SFHb or DCLHb (fig. 5). In the small intestine blood flow was significantly increased following the infusion of either SFHb ($F(3,16) = 5.65$; $P = 0.009$) or DCLHb ($F(3,24) = 2.43$; $P = 0.05$) and returned to normal 60 min after administration. The vascular resistance in the small intestine was not affected by SFHb or DCLHb. The blood flow and vascular resistance in the caecum and large intestine were not altered by SFHb or DCLHb (fig. 5).

Effect of SFHb and DCLHb on the blood circulation to skin and musculo-skeletal system

SFHb significantly ($F(3,16) = 3.29$; $P = 0.04$) increased blood flow to the skin 15 min after infusion and DCLHb significantly ($F(3,24) = 2.79$; $P = 0.002$) increased blood flow to the skin 30 min after infusion. The vascular resistance in the skin was not affected by the

administration of DCLHb or SFHb. The blood flow to the musculoskeletal system was not altered following the infusion of either SFHb or DCLHb. The vascular resistance in the musculoskeletal system was not affected by SFHb, but was significantly ($F(3,24) = 6.13$; $P = 0.005$) increased at 15, 30 and 60 min after the administration of DCLHb (fig. 6).

Effect of SFHb and DCLHb on the regional distribution of cardiac output

The distribution of cardiac output was not affected in the brain, gastrointestinal tract, kidneys and skin following the infusion of either SFHb or DCLHb (fig. 7). Infusion of both SFHb ($F(3,16) = 3.06$; $P = 0.03$) and DCLHb ($F(3,24) = 2.94$; $P = 0.05$) significantly increased the percent cardiac output to heart. In the musculo-skeletal system the percent cardiac output was not affected by the infusion of SFHb, but a significant decrease in the percent cardiac output was observed with the infusion of DCLHb ($F(3,24) = 5.72$; $P = 0.004$) 15, 30 and 60 min (fig. 7) after infusion.

3.4 Discussion

DCLHb infusion has been shown to be associated with an increase in the mean arterial pressure similar to that demonstrated with other haemoglobin solutions (Hamilton *et al.*, 1992; Sharma and Gulati, 1994; Schultz *et al.*, 1993). It is not known whether the pressor effect is associated with changes in regional blood circulation. The present study was an attempt to determine the effect of purified, modified and stabilized haemoglobin solution, DCLHb, on the regional circulation and compare it with an unmodified SFHb solution. It was found that there were clear differences in the regional circulatory and systemic hemodynamic effects of DCLHb and SFHb. Since the effect of SFHb and DCLHb on blood pressure, stroke volume and cardiac output were different, it was of interest to study the differences in the regional blood circulation due to the infusion of SFHb and DCLHb. The results of the present study indicate that DCLHb either increased the blood flow to several organs or did not affect blood flow. On the other hand, SFHb produced a significant decrease in blood flow to the kidneys, liver and spleen while DCLHb did not. The differences in the effect of unmodified (SFHb) and modified (DCLHb) haemoglobin solutions on the regional blood flow have been clearly demonstrated. The differences in the cardiovascular effects of SFHb and DCLHb solutions can be attributed to several possibilities. It could be due to (1) significantly high amounts of phospholipids (Feola *et al.*, 1988) present in SFHb as compared to DCLHb (table 1), (2) increased ability of SFHb to initiate an inflammatory reaction as compared to DCLHb (Burhop *et al.*, 1992), (3) increased ability of SFHb to release endogenous vasoconstrictor substances (eg. endothelin) as compared to DCLHb, (4) increased ability of SFHb to block endogenous vasodilator substances (eg. EDRF/nitric oxide) and/or (5) a combination of the above factors.

Unmodified SFHb has been reported to produce a transient increase in blood pressure and increase in blood flow to brain, heart and GIT (Ning *et al.*, 1992). Similar results were obtained in the present study. Ning et al. also observed that SFHb increased blood flow to the liver and kidneys (Ning *et al.*, 1992), while we found that blood flow was decreased in the liver and kidneys. Both studies used radioactive microspheres to determine regional blood flow, however, we used rats and the SFHb was administered as a volume load, while Ning et al. used dogs and the haemoglobin was administered in an exchange transfusion procedure (Ning *et al.*, 1992). The differences in the two studies could be attributed to species and administration of SFHb. Although earlier studies using isolated tissues and *in vitro* techniques had shown that SFHb produces coronary vasoconstriction (Vogel *et al.*, 1986), the present study using an *in vivo* technique, showed no evidence of coronary vasoconstriction. Similarly, *in vitro* studies have shown that haemoglobin is a vasoconstrictor for the cerebral blood vessels (Sonobe and Suzuki, 1978; Kajikawa *et al.*, 1979; Wellum *et al.*, 1980), but the present study showed no evidence of vasoconstriction of cerebral blood vessels. These differences may be attributed to the fact that in the *in vitro* situation the haemoglobin is acting on both luminal and abluminal sides of the cerebral blood vessels, while in the *in vivo* situation, where intravenous infusion is performed, haemoglobin may not act on the abluminal side, but rather is acting primarily on the luminal side of the cerebral blood vessels. If cardiovascular actions of haemoglobin are mediated through endogenous vasoactive substances, their presence in the *in vivo* situation and absence in the *in vitro* situation could be responsible for the differences in the vascular reactions of haemoglobin.

DCLHb did not decrease the blood flow to any organ including the kidneys, liver and spleen, whereas SFHb decreased the blood flow to these organs. It appears that modification of haemoglobin in the form of DCLHb may alter the vascular activity of haemoglobin. The vascular resistance which is primarily dependent upon the diameter of the blood vessel was increased in several organs by SFHb. On the other hand, DCLHb did not affect the vascular resistance in any major organ systems, except in the musculo-skeletal system in which DCLHb produced a significant increase. It appears that SFHb increased the blood pressure as a result of vasoconstriction in organs like the liver, spleen, kidneys, skin, mesentery and pancreas, while DCLHb increased the blood pressure due to vasoconstriction of blood vessels in the musculo-skeletal system. Differences in the ability of SFHb and DCLHb to initiate an inflammatory reaction have been demonstrated (Burhop *et al.*, 1992). Plasma concentrations of C3a and thromboxane B_2 were significantly increased with infusion of SFHb, while DCLHb infusion did not elicit any increase in C3a or thromboxane B_2 in sheep (Burhop *et al.*, 1992). It has been suggested that the presence of excess amounts of aminophospholipids in SFHb solution could be responsible for the inflammatory reaction (Feola *et al.*, 1988).

In conclusion, the present study provides evidence that the blood vessels in different organs do not show a similar response to different haemoglobin solutions. In addition, modified haemoglobin solutions affect the blood vessels in various organs differently than unmodified SFHb. It is not known whether the vascular actions of SFHb or DCLHb are (1) due to their direct action on the blood vessels, (2) are mediated through endogenous vasoactive substances like nitric oxide, endothelin, catecholamines, angiotensin etc and/or (3) due to the presence of excess amounts of phospholipids in SFHb. Studies are in progress to determine the role of other vasoactive substances in the regional circulatory effects of DCLHb.

3.5 Acknowledgments

The authors would like to thank (1) Dr. P.R. Saxena from Erasmus University, Rotterdam, The Netherlands for providing the software for calculations involved in radioactive microsphere technique.

Chapter 4

Effect of diaspirin crosslinked haemoglobin and norepinephrine on systemic hemodynamics and regional circulation in rats

Summary

Diaspirin crosslinked haemoglobin (DCLHb™) (400 mg/kg, i.v.) produced a pressor effect which was equal to that produced by norepinephrine (NE) (25 μg/kg/min i.v. infusion). Total peripheral resistance was increased by DCLHb and more significantly by NE. Heart rate was not affected by DCLHb but was significantly increased by NE. The cardiac output and stroke volume were insignificantly increased by DCLHb but were significantly decreased by NE. DCLHb and NE produced a significant increase in blood flow to the heart. The vascular resistance in the heart was not affected by DCLHb but was decreased by NE. DCLHb did not affect the renal and brain circulation but NE in kidneys decreased the blood flow and increased the vascular resistance, while in brain increased the blood flow and decreased the vascular resistance. DCLHb increased the blood flow to the stomach and small intestine. The vascular resistance was not affected by DCLHb in the gastrointestinal tract. NE did not affect the blood circulation in the gastrointestinal tract. The blood flow to spleen was increased by DCLHb and there was no change in the vascular resistance. NE insignificantly decreased the blood flow to spleen and significantly increased the vascular resistance. The blood circulation to the mesentry and pancreas was not affected by DCLHb, while NE increased the blood flow without affecting the vascular resistance. DCLHb produced a significant increase in the blood flow to the skin without affecting the vascular resistance, while NE did not affect the blood flow but increased the vascular resistance. DCLHb did not affect the blood flow to musculo-skeletal system but increased the vascular resistance, while NE decreased the blood flow and increased the vascular resistance. In summary, although the pressor effect of DCLHb and NE at the doses studied is equal, DCLHb did not decrease the blood flow to any organ, whereas NE produced significant decreases in blood flow to several organs. It is concluded that the blood flow to most of the organs is either increased or not affected by DCLHb.

4.1 Introduction

Haemoglobin solutions are being developed as promising resuscitative solutions. Investigators have stressed several advantages of haemoglobin solutions as compared to other resuscitating solutions (Bonhard, 1975; Kaplan and Murphy, 1975; Moss *et al.*, 1976; DeVenuto and Zegna, 1978). The effect of haemoglobin solutions on the vascular activity has been of interest for decades. The first investigation was performed by Brodie, who found that intravenous administration of hemolysate into cats produces hypotension and bradycardia (Brodie, 1990). Subsequently, using a more purified haemoglobin saline solution, it was found

that in cats the initial hypotension was followed by an increase in blood pressure (Amberson *et al.*, 1934) which could be due to the presence of a pressor substance (Amberson *et al.*, 1949). Several studies have been performed demonstrating cardiovascular actions of stroma free haemoglobin solutions. Purified stroma free haemoglobin solutions have been reported to produce vasoconstrictor activity in a variety of experimental models (Wellum *et al.*, 1980; Vogel *et al.*, 1986; Gilroy *et al.*, 1988). Haemoglobin has been shown to be a vasoconstrictor for cerebral arteries (Sonobe and Suzuki, 1978; Kajikawa *et al.*, 1979). Yamakawa et al. reported that stroma free haemoglobin solution administration produced a marked vasodilatation of coronary blood vessels in the rat (Yamakawa *et al.*, 1990). Unmodified human stroma-free haemoglobin was found to produce an increase in the blood flow to the heart, brain, liver, gut and kidneys of dog (Ning *et al.*, 1992). It has not been clear whether it is the haemoglobin molecule per se or some unknown contaminant which is responsible for either vasoconstriction or vasodilation.

Diaspirin crosslinked haemoglobin (DCLHb) is a blood substitute derived from the haemoglobin of outdated erythrocytes by crosslinking the haemoglobin between the α-subunits by reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process includes heat pasteurization of the solution, as described earlier (Estep *et al.*, 1989b; Estep *et al.*, 1989a). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). The cross-linking of the α subunits results in a haemoglobin with a favorable oxygen dissociation curve (Snyder *et al.*, 1987; Vandegriff *et al.*, 1989). It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions were not affected significantly (Hess *et al.*, 1989). DCLHb has been found to be a promising resuscitative fluid following hemorrhage (Przybelski *et al.*, 1990). DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters (Przybelski *et al.*, 1991). DCLHb has also been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion in rats (Cole *et al.*, 1992).

DCLHb increases the mean arterial pressure when administered to hemorrhagic (Przybelski *et al.*, 1991) or normal rats (Hamilton *et al.*, 1992). Other haemoglobin solutions have also been reported to produce a rapid and sustained increase in mean arterial pressure (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). It is not known whether DCLHb affects the regional blood flow to various organs. Norepinephrine (NE), an extensively studied pressor agent, is known to produce significant effect on the blood circulation in several organs. It could be possible that DCLHb which increases the blood pressure might be affecting the regional blood circulation. The present study was conducted to determine the effect of DCLHb on regional

circulation and to compare its effect with NE. The dose of NE was selected so that its pressor effect was similar to DCLHb.

4.2 Materials and Methods

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 300-350 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure. In order to keep the blood pO_2, pCO_2 and pH constant and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683. The carotid artery of the right side was exposed and a PE 50 cannula guided through the common carotid artery to the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P 23 DC pressure transducer. When the cannula reached the left ventricle, the diastolic pressure dropped to zero. The femoral artery of the right side was also cannulated and connected to a withdrawal pump (Harvard Model 22).

At each measurement, a thoroughly mixed suspension of approximately 200,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA) in 0.2 ml saline was injected into the left ventricle and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all tissue and organs were dissected out, weighed and placed in vials containing 10% formalin. The following tissues were studied: lungs, heart, liver, stomach, small intestine, caecum, large intestine, mesentry and pancreas, spleen, left kidney, right kidney, left cerebral hemisphere, right cerebral hemisphere, midbrain, cerebellum, brain stem, skin and rest of the body consisting of muscles and bones. The radioactivity in the standards, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output (2) stroke volume (3) total peripheral resistance (4) regional blood flow and (5) regional vascular resistance. These parameters were calculated using the programs described earlier (Saxena *et al.*, 1980). Infusion of saline did not affect the systemic hemodynamics and regional circulation significantly. The effect of DCLHb (400

mg/kg, i.v.) and NE (25 μg/kg/min i.v. infusion for 60 min) on systemic hemodynamics and regional circulation was studied. The doses used in tissue blood flow studies were selected so that a similar pressor response was produced by DCLHb and NE.

Drugs and solutions

DCLHb (10 % solution was infused in the volume of 4 ml/kg) was prepared and provided by Baxter Healthcare Corp., Round Lake, IL. NE was purchased from Sigma Chemical Co., St. Louis, MO and administered in the volume of 4 ml/kg.

Statistics

All data are presented as the mean values ± 1 SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Duncan's test. A level of $P < 0.05$ was considered significant.

4.3 Results

Effect of saline, DCLHb and NE on systemic hemodynamics of rats

DCLHb (400 mg/kg, i.v.) produced an increase ($F(3,20) = 11.28$; $P = 0.0001$) in blood pressure similar to that caused by NE (25 μg/kg/min i.v. infusion) ($F(3,40 = 10.80$; $P = 0.0001$) (Table 1). The total peripheral resistance (TPR) increased significantly ($F(3,20) = 2.72$; $P = 0.0407$), only 60 min after the administration of DCLHb. NE significantly increased the TPR ($F(3,40) = 5.82$; $P = 0.0021$) as compared to DCLHb. DCLHb did not affect the heart rate. NE significantly increased ($F(3,40) = 5.49$; $P = 0.0031$) the heart rate 15, 30 and 60 min of infusion. The cardiac output and stroke volume were insignificantly increased with DCLHb. However, NE produced a significant decrease in cardiac output at 60 min ($F(3,40) = 2.84$; $P = 0.004$) and stroke volume at 15, 30 and 60 min ($F(3,40) = 5.19$; $P = 0.004$) of infusion (Table 1). The infusion of an equal volume of saline did not produce any significant effect on the blood pressure, heart rate, TPR, cardiac output and stroke volume (Table 1).

Effect of saline, DCLHb and NE on the coronary blood circulation

DCLHb produced a significant increase ($F(3,20) = 4.06$; $P = 0.02$) in the blood flow to the heart (Fig. 1). The vascular resistance decreased, but not significantly, with DCLHb administration. NE produced a significant increase ($F(3,40= 13.00$; $P < 0.0001$) in the blood flow to the heart along with a significant decrease ($F(3,40) = 4.24$; $P = 0.01$) in the vascular resistance. Saline, when administered in equal volume, did not affect the blood flow or vascular resistance in the heart (Fig. 1).

Effect of saline, DCLHb and NE on the renal blood circulation

Blood flow and vascular resistance in the left and right kidneys were not affected by DCLHb (Fig. 2). On the other hand, NE produced a significant decrease in the blood flow to both left ($F(3,40) = 3.39$; $P = 0.02$) and right ($F(3,40) = 3.96$; $P = 0.01$) kidneys at 15, 30 and 60 min of infusion. The vascular resistance increased following NE administration to a statistically significant degree ($F(3,40) = 2.57$; $P = 0.016$) at 60 min of infusion (Fig. 2). Saline did not produce any change in either renal blood flow or in the vascular resistance (Fig. 2).

Effect of saline, DCLHb and NE on the brain blood circulation

DCLHb and saline did not affect the blood flow and vascular resistance in either cerebral hemisphere, the diencephalon, cerebellum or brain stem. NE infusion produced a significant increase ($F(3,40) = 4.88$; $P = 0.0055$) in the blood flow to the cerebral hemispheres, diencephalon ($F(3,40) = 3.25$; $P = 0.03$), cerebellum ($F(3,40) = 4.06$; $P = 0.013$) and brain stem ($F(3,40) = 4.14$; $P = 0.012$) at 15, 30 and 60 min of infusion (Fig. 3). The vascular resistance decreased significantly ($F(3,40) = 4.57$; $P = 0.008$) in the cerebral hemispheres at 15, 30 and 60 min, cerebellum ($F(3,40) = 2.02$; $P = 0.0417$) at 30 min, and brain stem ($F(3,40) = 3.14$; $P = 0.036$) at 15, 30 and 60 min of infusion. The decrease in vascular resistance in the diencephalon was not statistically significant (Fig. 3).

Effect of saline, DCLHb and NE on the gastrointestinal tract (GIT) blood circulation

DCLHb produced a significant increase ($F(3,20) = 4.32$; $P = 0.016$) in the blood flow to the stomach at 15 and 30 min and small intestine ($F(3,20) = 2.45$; $P = 0.01$) at 30 min of infusion. The blood flow to the caecum and large intestine was not affected by DCLHb (Fig. 4). The vascular resistance was not changed in stomach, small intestine, caecum or large intestine. NE and saline did not affect the blood flow or the vascular resistance in any region of the GIT (Fig. 4).

Effect of saline, DCLHb and NE on the portal blood circulation

DCLHb did not produce a significant effect on the blood flow or vascular resistance in liver, mesentry and pancreas. However, DCLHb produced a marked increase ($F(3,20) = 2.89$; $P = 0.035$) in the blood flow to the spleen at 15 min of infusion. The vascular resistance was not affected in the spleen (Fig. 5). On the other hand, NE did not affect the blood flow or vascular resistance in liver and spleen. However, a significant increase ($F(3,40) = 2.65$; $P = 0.019$) in the blood flow occurred in the mesentry and pancreas at 15 min of NE infusion. The vascular resistance was not altered. Saline did not produce any significant effect on the blood circulation in the liver, spleen, mesentry and pancreas (Fig. 5).

Effect of saline, DCLHb and NE on the skin and musculo-skeletal blood circulation

Saline did not affect the blood circulation in the skin or musculo-skeletal system. DCLHb produced a significant increase ($F(3,20) = 2.70$; $P < 0.0305$) in the blood flow to skin but did not

affect the blood flow to musculo-skeletal system. The vascular resistance was not altered in skin, but a significant increase ($F(3,20) = 5.57$; $P = 0.001$) in the vascular resistance was observed in the musculo-skeletal system 15, 30 and 60 min following DCLHb administration (Fig. 6). On the other hand, NE produced no effect on the skin blood flow, but increased ($F(3,40) = 2.25$; $P = 0.04$) the vascular resistance in the skin. NE produced a significant decrease ($F(3,40) = 8.07$; $P = 0.0003$) in the blood flow and an increase ($F(3,40) = 7.67$; $P = 0.0005$) in the vascular resistance in the musculo-skeletal system (Fig. 6).

Table 1: Effect of saline (4 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N = 6) and NE (25 µg/kg/min; i.v. infusion; N = 11) on the systemic hemodynamics of anesthetized rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)				
Saline	350 ± 10	334 ± 11	340 ± 17	342 ± 17
DCLHb	386 ± 10	397 ± 10	398 ± 11	395 ± 10
NE	399 ± 7	454 ± 8*	453 ± 9*	435 ± 16*
Mean BP (mmHg)				
Saline	82 ± 4	77 ± 5	76 ± 5	78 ± 3
DCLHb	84 ± 6	150 ± 9*	144 ± 11*	125 ± 9*
NE	96 ± 3	143 ± 7*	146 ± 10*	127 ± 7*
Cardiac output (ml/min)				
Saline	82 ± 11	84 ± 14	75 ± 11	66 ± 8
DCLHb	82 ± 10	107 ± 7	119 ± 20	86 ± 6
NE	81 ± 4	94 ± 7	80 ± 14	42 ± 5*
Stroke Volume (ml)				
Saline	0.23 ± 0.02	0.24 ± 0.03	0.21 ± 0.02	0.19 ± 0.01
DCLHb	0.21 ± 0.02	0.26 ± 0.02	0.31 ± 0.06	0.22 ± 0.02
NE	0.28 ± 0.04	0.21 ± 0.02*	0.17 ± 0.03*	0.15 ± 0.02*
TPR (mmHg/l/min)				
Saline	1054 ± 123	981 ± 114	1071 ± 126	1265 ± 186
DCLHb	1002 ± 87	1452 ± 149	1347 ± 207	1570 ± 110*
NE	960 ± 104	1633 ± 154	2388 ± 411*	2287 ± 313*

*$p < 0.05$ as compared to control.

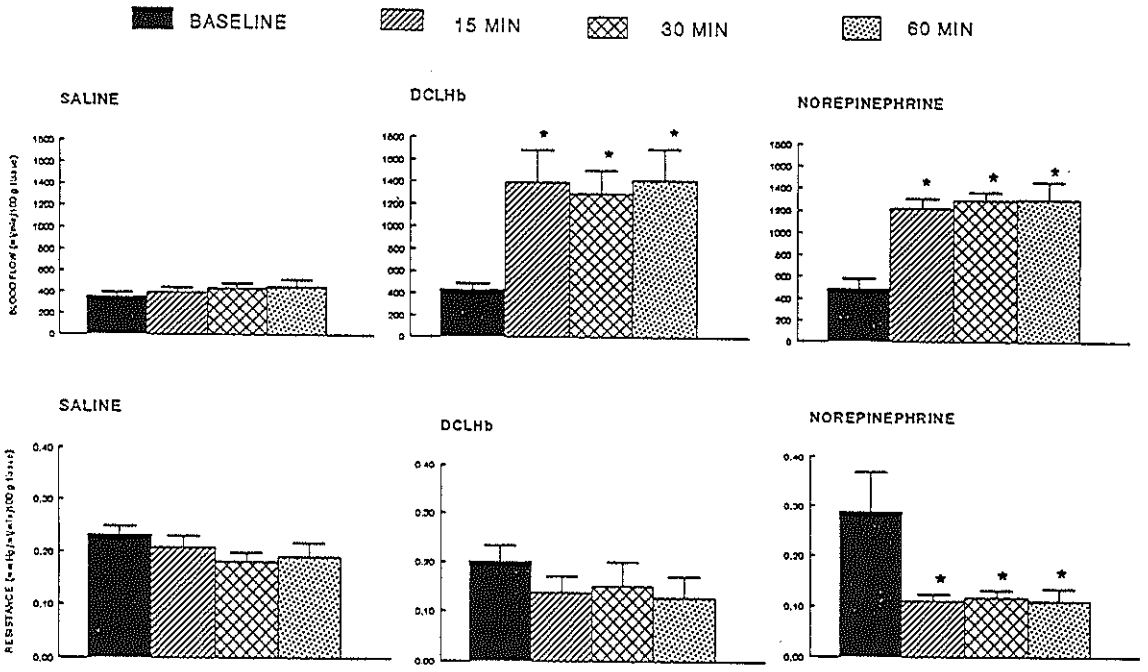

Fig. 1 The effect of saline (4.0 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N= 6) and NE (25 µg/kg, i.v..; N= 11) on the blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the heart. *Indicates significant difference as compared to baseline.

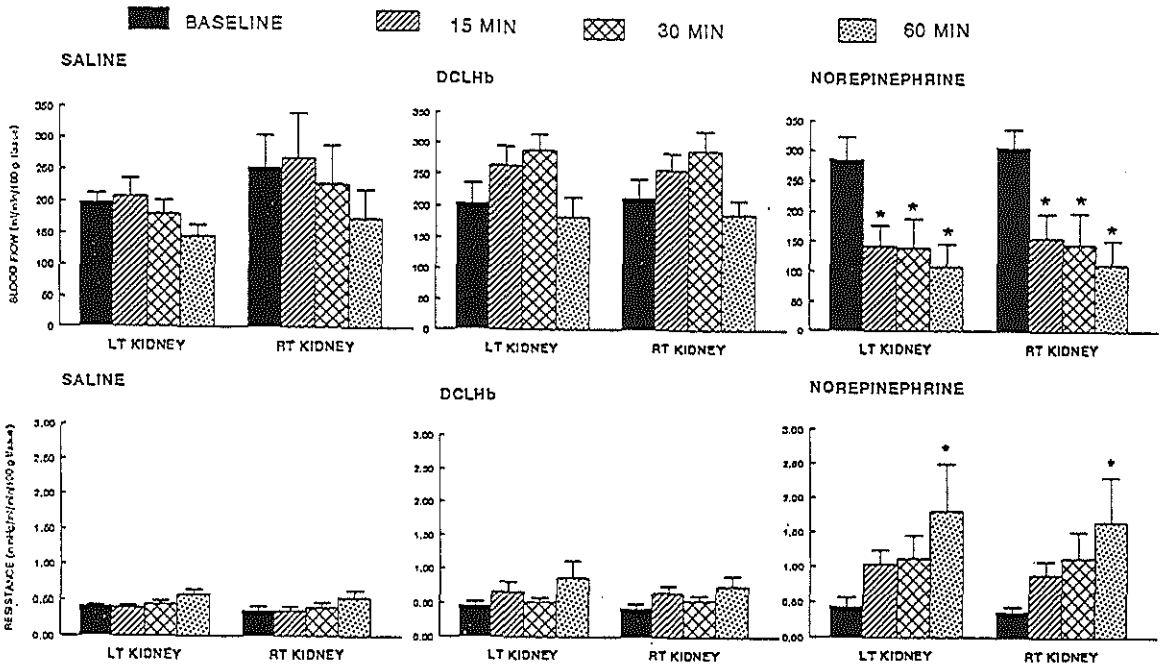

Fig. 2 The effect of saline (4.0 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N= 6) and NE (25 μg/kg, i.v..; N= 11) on the blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the left and right kidneys. *Indicates significant difference as compared to baseline.

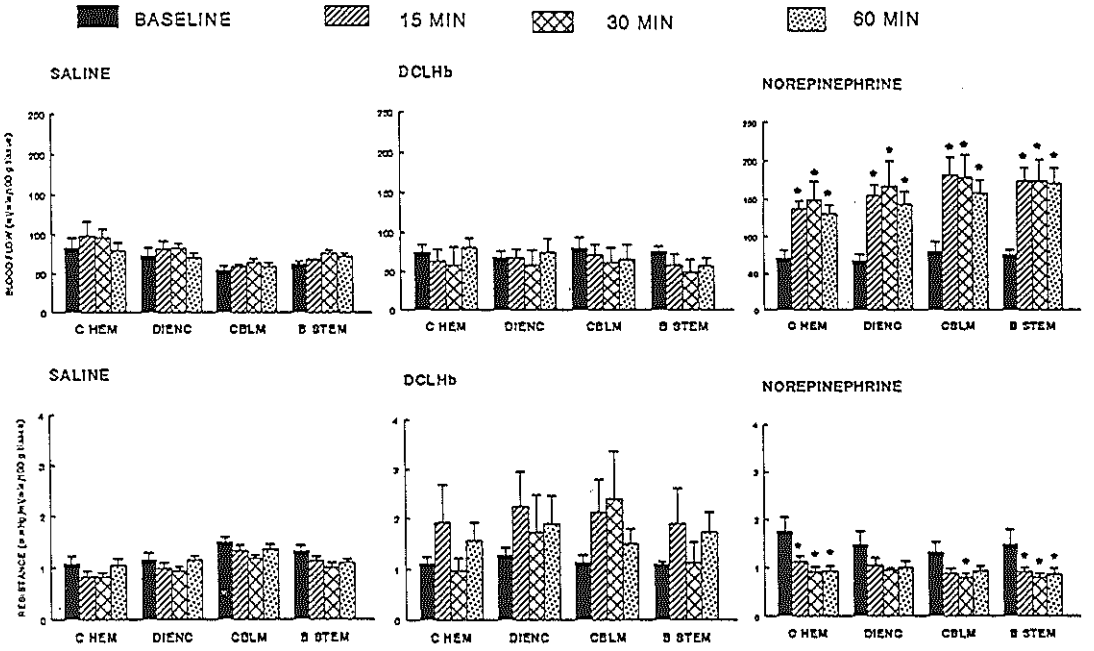

Fig. 3 The effect of saline (4.0 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N= 6) and NE (25 µg/kg, i.v..; N= 11) on the blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the cerebral hemispheres (C HEM), diencephalon (DIENC), cerebellum (CBLM) and brain stem (B STEM). *Indicates significant difference as compared to baseline.

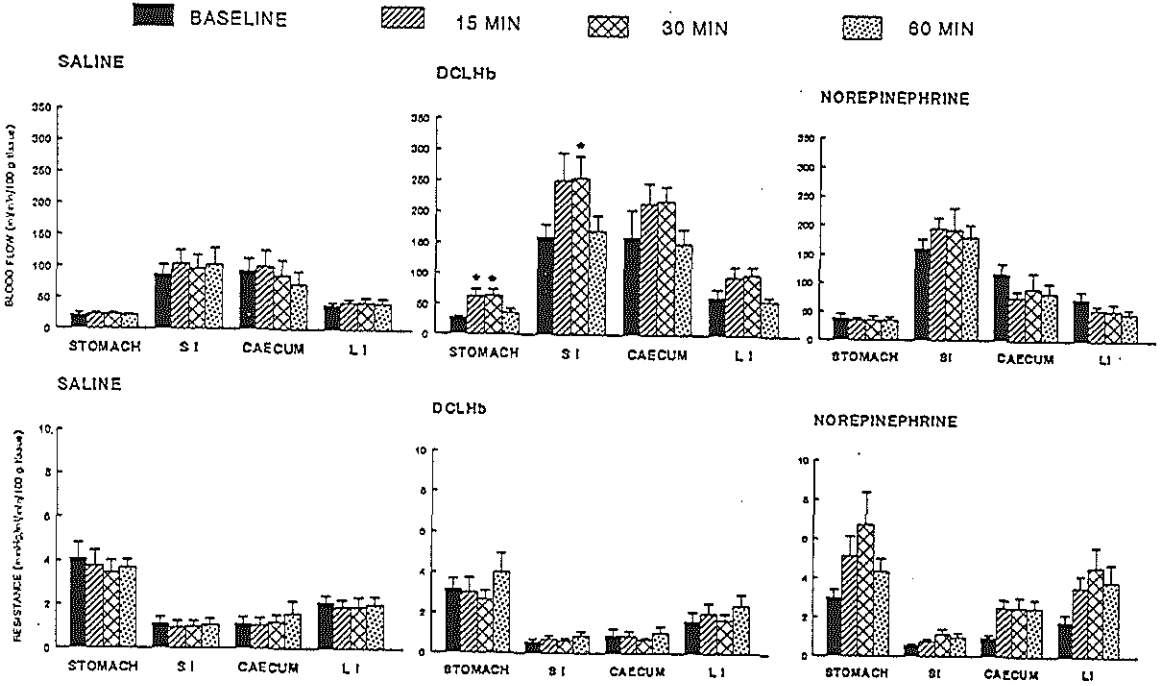

Fig. 4 The effect of saline (4.0 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N= 6) and NE (25 µg/kg, i.v..; N= 11) on the blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the stomach, small intestine (SI), caecum and large intestine (LI). *Indicates significant difference as compared to baseline.

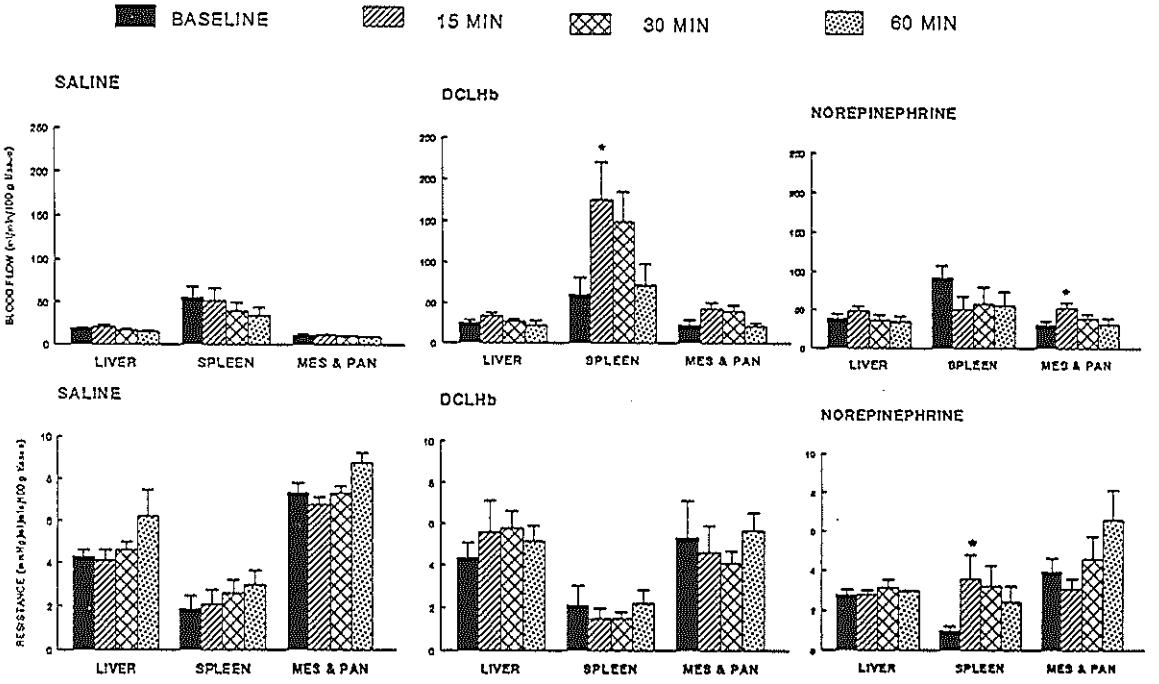

Fig. 5 The effect of saline (4.0 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N= 6) and NE (25 µg/kg, i.v..; N= 11) on the blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the liver, spleen, mesentry and pancreas. *Indicates significant difference as compared to baseline.

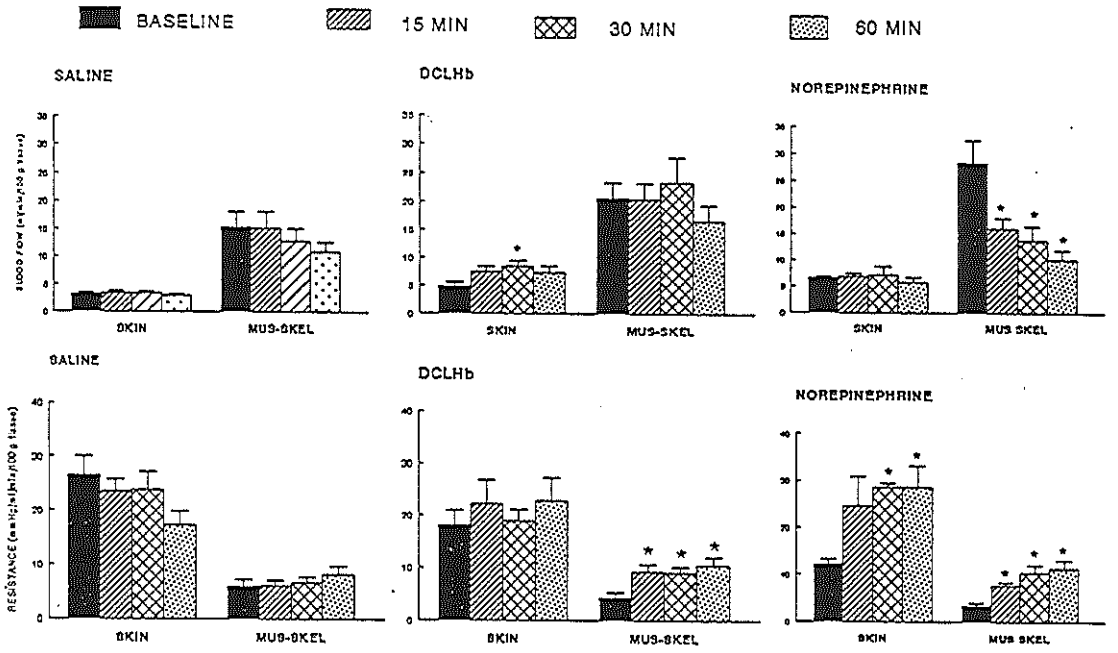

Fig. 6 The effect of saline (4.0 ml/kg, i.v.; N = 5), DCLHb (400 mg/kg, i.v.; N= 6) and NE (25 µg/kg, i.v.; N= 11) on the blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the skin and musculo-skeletal system. *Indicates significant difference as compared to baseline.

4.4 Discussion

The hemodynamic response to blood replacement with stroma-free haemoglobin differs from that observed with other fluids (Vogel *et al.*, 1986) and has been attributed to improved systemic oxygen delivery (Hauser *et al.*, 1982). However, the vascular response to stroma-free haemoglobin is believed to be independent of oxygen affinity (Nees *et al.*, 1978). Stroma-free haemoglobin solutions have been reported to produce a pressor effect (Przybelski *et al.*, 1991; Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989; Kida *et al.*, 1991), which could be due to the vasoconstriction of blood vessels in different regions. The present study was conducted to determine whether DCLHb, which produces a pressor response in rats and several other species, affects the blood flow to different organs/tissues.

The present study provides additional information on the pressor effect of DCLHb in rats. A dose of NE (25 μg/kg, i.v. infusion) was selected which produced the same degree of pressor response as DCLHb (400 mg/kg, i.v.). TPR increased with DCLHb and NE but the increase in TPR was more marked with NE. NE increased the heart rate but DCLHb had no effect. Cardiac output and stroke volume were insignificantly increased by DCLHb but were markedly decreased by NE. It appears that the pressor effect of NE is primarily due to the increase in TPR resulting from vasoconstriction of the blood vessels. On the other hand, the pressor effect of DCLHb appears to be due to a combination of an increase in the TPR (41%) and in the cardiac output (42%). DCLHb increased the blood flow to heart, stomach, small intestine, spleen and skin, while NE increased the blood flow to heart, cerebral hemispheres, diencephalon, cerebellum, brain stem, mesentry and pancreas. DCLHb did not decrease the blood flow to any organ, but NE decreased the blood flow to kidneys and the musculo-skeletal system. These observations suggest that regional circulatory effects of DCLHb and NE are different. It could be possible that the cardiovascular effect of NE are mediated through the α adrenoceptors, while those of DCLHb are mediated through α adrenoceptors and/or other mechanisms.

The results of the present study clearly demonstrate that the pressor effect of DCLHb does not indicate a decrease in blood flow to various organs/tissues. Instead, the blood flow was found to be increased in several organs. The increase in blood flow by DCLHb appears to be due to redistribution of the cardiac output. An increase in the vascular resistance in the musculo-skeletal system by DCLHb appears to be responsible for the redistribution of blood flow from the musculo-skeletal system. Since about 54% of the cardiac output in the rat goes to the musculo-skeletal system (personal observation) a small decrease in the percent cardiac output to the musculo-skeletal system will lead to a marked increase in the blood flow to other organs receiving smaller percentage of cardiac output. Increase in the blood flow to vital organs by DCLHb might be an additional advantage in clinical situations involving resuscitation.

The results of the present study are consistent with some of the studies conducted earlier. Ultrapurified, polymerized bovine haemoglobin when administered in rats produced a marked increase in blood pressure and systemic vascular resistance, while the stroke volume remained unchanged (Waschke *et al.*, 1993). DCLHb similarly increased the blood pressure and vascular resistance but had no effect on stroke volume. Stroma-free, polymerized and pyridoxylated haemoglobin administered to calves produced an increase in blood pressure (Schistek *et al.*, 1992) but the blood flow was not measured in this study. Unmodified human stroma-free haemoglobin solution when administered to dogs produced a pressor effect, but the cardiac output remained unchanged. Blood flow was found to increase in the heart, brain, liver, gut and kidney 30 min after the administration of haemoglobin solution. The glomerular filtration rate decreased and the urine flow was reduced (Ning *et al.*, 1992). Since the blood flow to the kidneys is increased and there is no change in cardiac output, it is difficult to explain the factors responsible for the decrease in the glomerular filtration rate. In the present study the cardiac output was not significantly altered by DCLHb and the blood flow was increased in the heart and GIT, but not in the liver, brain and kidneys. The reasons for these differences could be due to (1) the species used, we used rats while they used dogs, (2) the type of haemoglobin, the molecular modification of haemoglobin could be responsible for altered hemodynamic responses, (3) the method of haemoglobin administration, ours were top loaded and Ning and co-workers did isovolumic exchange transfusion, and (4) the purity of haemoglobin solutions.

The biochemical mechanisms involved in the vascular actions of haemoglobin solution have not been studied extensively. In one of the few studies which have been performed, the constrictor responses to angiotensin II and 5-HT after exchange transfusion with pyridoxalated haemoglobin polyoxyethylene conjugate (PHP) were not found to be altered (Kida *et al.*, 1991). However, the responses to NE were significantly augmented in PHP transfused rats. Kida et al. also found that PHP does not affect the vasodilator actions of acetylcholine and nitroglycerine (Kida *et al.*, 1991). We have also found that DCLHb increases the sensitivity of vascular α adrenoceptors (Gulati and Rebello, 1994). Prazosin, an α adrenoceptor blocker, has been found to decrease the pressor effect induced by DCLHb (Bilello *et al.*, 1994). It appears that the cardiovascular actions of DCLHb are at least in part mediated through the α adrenoceptors. Since the hemodynamic response of DCLHb is different from that of NE, it appears that DCLHb may also be acting through a different mechanism. Phosphoramidon, an inhibitor of pro-endothelin conversion to endothelin, attenuated the elevation of blood pressure by DCLHb in rats indicating that the pressor effect of DCLHb might be mediated through endothelin mechanisms (Schultz *et al.*, 1993). L-arginine, the substrate for NO and nitroglycerine, a NO donor, significantly reduced the pressor effect of DCLHb, when infused in rats 15 min after the administration of DCLHb (Schultz *et al.*, 1993). These studies clearly indicate that NO system

is also involved in the pressor effect of DCLHb. It could be possible that multiple mechanisms are involved in the cardiovascular actions of DCLHb.

It can be concluded that: (1) The pressor effect of DCLHb is not associated with a decrease in blood flow to various organs and, in fact, increases in some vascular beds. (2) Although the pressor effect of NE and DCLHb were similar, NE produced marked hemodynamic changes, including a decrease in blood flow to the kidneys, whereas DCLHb did not. (3) The increase in blood flow induced by DCLHb to the heart, spleen, GIT and skin appears to be due to the redistribution of blood flow.

4.5 Acknowledgments

The authors would like to thank (1) Dr. P.R. Saxena from Erasmus University, Rotterdam, The Netherlands for providing the software for the calculations involved in radioactive microsphere technique, (2) Baxter Healthcare Corp. for providing financial assistance.

Part 3

Role of adrenergic mechanism in the cardiovascular effects of diaspirin crosslinked haemoglobin

Chapter 5

Role of adrenergic mechanisms in the pressor effect of diaspirin cross-linked haemoglobin

Summary

Diaspirin cross-linked haemoglobin (DCLHb™; Baxter Healthcare Corp.) is a promising haemoglobin based oxygen carrying resuscitative solution. DCLHb (400 mg/kg, i.v.) produces an immediate increase in blood pressure when administered to conscious or anesthetized rats. To determine the role of the central nervous system (CNS) and the peripheral vascular system in the pressor effect of DCLHb, experiments were performed in cervical sectioned rats. Intravenous administration of DCLHb produced an increase in blood pressure in cervical sectioned animals (41.2 ± 2.5 mmHg), which was similar to that observed in normal rats. To test whether the pressor effect was due to release of catecholamines or other pressor substances from the adrenal medulla, DCLHb was administered to bilateral adrenal demedullated rats. It was found that DCLHb produced a pressor effect in bilateral adrenal demedullated rats (42.0 ± 6.4 mmHg) that was similar to normal rats. To determine whether DCLHb alters the responsiveness of peripheral vascular adrenoceptors, the effect of DCLHb pretreatment on the blood pressure and heart rate responses of adrenergic agonists was studied. DCLHb significantly potentiated (66.7%) the pressor response to norepinephrine (0.5 to 2.0 μg/kg, i.v.) but did not affect the heart rate response to norepinephrine. Phenoxybenzamine completely blocked the DCLHb induced potentiation of the norepinephrine responses. Phenylephrine produced a dose dependent (5 to 20 μg/kg, i.v.) pressor and reflex bradycardic effect. DCLHb significantly potentiated the pressor (40.6%) and bradycardic (-22.8%) effect of phenylephrine which were completely blocked by prazosin. Clonidine produces a fall in blood pressure by acting on the central α-adrenoceptors, and a rise in blood pressure by stimulating the peripheral vascular α-adrenoceptors. DCLHb produced a marked potentiation of the pressor response to clonidine (75 μg/kg, i.v.) that masked the central depressor effect. Prazosin pretreatment did not attenuate the DCLHb induced potentiation of the pressor effect of clonidine in intact rats. However, yohimbine pretreatment completely blocked the DCLHb induced potentiation of the clonidine induced pressor response in intact rats. In order to exclude the contribution of a centrally induced cardiovascular effect of clonidine, further studies were carried out in cervical sectioned rats. DCLHb markedly potentiated the pressor effect of clonidine (25 μg/kg, i.v.) in cervical sectioned rats. This potentiation could be attenuated by prazosin and yohimbine. Pretreatment with either yohimbine (2 mg/kg, i.v.) or prazosin (1 mg/kg, i.v.) significantly attenuated the DCLHb induced pressor effect. The attenuation of DCLHb induced pressor effect was more marked with prazosin as compared to yohimbine. It is concluded that the pressor effect of DCLHb is not mediated through the CNS, however, it appears that in rats both α_1- and α_2-adrenoceptors in the peripheral vascular system are sensitized by DCLHb.

5.1 Introduction

Considerable research has been performed to develop and evaluate blood substitutes as resuscitative solutions. Resuscitation solutions can be non-oxygen carriers (plasma, dextran and albumin) or oxygen carriers (haemoglobin solutions, perfluorochemicals and synthetic chelates). Many investigators have stressed several advantages of haemoglobin solutions as compared with other resuscitating solutions (Kaplan and Murphy, 1975; Moss *et al.*, 1976; Bonhard, 1975; DeVenuto and Zegna, 1978). Several approaches have been made to reduce the toxicity and improve the oxygen delivery of haemoglobin based blood substitutes. Chemical modifications like cross-linking, polymerization and conjugation of haemoglobin has been performed. Encapsulated haemoglobin and haemoglobin emulsions have also been prepared. A blood substitute derived from the haemoglobin of outdated erythrocytes has been developed by cross-linking molecular haemoglobin between the α-subunits by reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process includes heat pasteurization of the solution (Estep *et al.*, 1989a; Estep *et al.*, 1989b). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). The cross-linking of the α subunits affords the haemoglobin a favorable oxygen dissociation curve (Snyder *et al.*, 1987; Vandegriff *et al.*, 1989).

It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions are not affected significantly (Hess *et al.*, 1989). DCLHb was also found to be a promising resuscitative fluid after hemorrhage (Przybelski *et al.*, 1990). Resuscitation with DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters (Przybelski *et al.*, 1991). DCLHb has been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion in rats (Cole *et al.*, 1992).

Haemoglobin solutions have been reported to produce a rapid and sustained increase in mean arterial pressure (Jesch *et al.*, 1982; Messmer *et al.*, 1977; Rabinovici *et al.*, 1989). DCLHb increases the mean arterial pressure when administered in hemorrhagic rats (Przybelski *et al.*, 1991). DCLHb (125-4000 mg/kg), when administered intravenously produced a 25-35 % increase in mean arterial pressure in rats. The pressor effect was self limiting and was not due to volume load or oncotic pressure (Hamilton *et al.*, 1992). The mechanism(s) responsible for the increase in blood pressure following DCLHb administration remain to be elucidated. The increase in blood pressure by DCLHb could be due to modification in one or more of several factors regulating blood pressure, such as endothelium derived relaxing factor (EDRF)/ nitric oxide (NO), endothelin, renin-angiotensin, or adrenergic agents. In the present study we have determined the role of adrenergic mechanisms in the pressor effect of DCLHb.

5.2 Materials and Methods

Animals

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 300-350 g were housed three to a cage, in a room with controlled temperature (23 ± 1 °C), humidity (50 ± 10%) and artificial light (0600-1800 hr). The animals were given food and water continuously. The experiments were begun only after the animals were acclimatized to the environment.

Drugs

Clonidine, norepinephrine, phenylephrine, yohimbine and prazosin were purchased from Sigma Chemical Co., St. Louis, MO. Phenoxybenzamine was obtained from Smith Kline and French Labs., Philadelphia, PA. Diaspirin cross-linked haemoglobin in lactated electrolyte was provided by Baxter Healthcare Corporation, Round Lake, IL. All the drugs were prepared in normal saline, except yohimbine, which was dissolved in ethanol and diluted with normal saline, and prazosin, which was dissolved in propylene glycol and diluted with normal saline. The drugs were prepared fresh at the time of each experiment.

Determination of blood pressure and heart rate

Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683.

Cervical section

In order to investigate whether the pressor effect of DCLHb is mediated through the CNS or through the peripheral vascular system, experiments were performed in cervical sectioned rats. To accomplish this, the rats were prepared as described above for measurement of blood pressure and heart rate. A dorsal midline incision was made and the vertebral muscles were separated and retracted to expose the occipital bone and cervical vertebrae. The spinous process and lamina of the upper cervical vertebrae were dissected out and removed to expose the spinal cord. The spinal cord was sectioned in the rats at the cervical level and a part of the cervical spinal cord was removed so that there was absolutely no connection with the brain. A period of one hour was allowed for stabilization and then the drugs were administered.

Bilateral adrenal demedullation

These experiments were performed to investigate whether the pressor effect of DCLHb could be due to the release of catecholamines or other pressor substances from the adrenal medulla. The bilateral adrenal demedullation was performed as described earlier (Borkowski and Quinn, 1983; Gulati and Bhargava, 1988). Briefly, the animals were anesthetized with sodium pentobarbitone (40 mg/kg, i.p.), a single midline incision was made, and the adrenal gland of one side was exposed, a small incision was made in the cortex and the medulla was enucleated by gently squeezing the gland with the help of a small blunt forcep. Similarly, the adrenal medulla on the other side was removed. The abdomen was closed in layers. Strict aseptic conditions were used throughout the surgical procedure. Seven days after surgery the rats were used for experiment. Sham treated animals underwent surgery exactly the same way except the adrenal medulla was not removed.

Interaction of DCLHb with adrenergic agents

Specific and nonspecific α_1- and α_2-adrenoceptor agonists and antagonists were used to determine the specificity and the subtypes of α-adrenoceptors involved in the pressor effect. Norepinephrine, which acts on both α_1 and α_2 adrenoceptors was administered in (1) untreated (control rats), (2) 15 min after the administration of DCLHb (DCLHb treated rats), and (3) 15 min after the administration of DCLHb and phenoxybenzamine (an α_1 and α_2 adrenoceptor antagonist) (DCLHB and phenoxybenzamine treated rats). Phenylephrine, a specific α_1 adrenoceptor agonist, was administered in (1) untreated (control rats), (2) 15 min after the administration of DCLHb (DCLHb treated rats) and (3) 15 min after the administration of DCLHb and prazosin (a specific α_1 adrenoceptor antagonist) (DCLHb and prazosin treated rats). Clonidine, which acts mainly on α_2 adrenoceptors and to a less extent on α_1 adrenoceptors, was administered in (1) untreated (control rats), (2) 15 min after the administration of DCLHb (DCLHb treated rats), (3) 15 min after the administration of DCLHb and yohimbine (a specific α_2 adrenoceptor antagonist) (DCLHb and yohimbine treated rats) and (4) 15 min after the administration of DCLHb and prazosin (a specific α_1 adrenoceptor antagonist) (DCLHb and prazosin treated rats). The effect of clonidine was studied in intact and cervical sectioned rats.

Statistics

All data are presented as the mean values ± 1 SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Scheffe's S test. A level of $P < 0.05$ was considered significant.

5.3 Results

Effect of DCLHb in cervical sectioned rats

In intact control rats, DCLHb (400 mg/kg, i.v.) was found to produce an increase in blood pressure and an insignificant decrease in heart rate. In cervical sectioned rats, the pressor effect of DCLHb was significantly ($F(1,8) = 12.42$; $P = 0.006$) greater, while the decrease in heart rate was similar ($F(1,8) = 0.01$; $P = 0.922$) to the intact control rats (fig. 1). The pressor effect of DCLHb in cervical sectioned rats, however, lasted only for about 60-90 min compared to 150 min in intact control rats.

Effect of DCLHb in bilateral adrenal demedullated rats

The pressor effect and the heart rate response to DCLHb (400 mg/kg, i.v.) in bilateral adrenal demedullated rats was found to be similar to that observed in intact control rats (fig. 2).

Effect of DCLHb on norepinephrine induced cardiovascular responses

The effect of DCLHb pretreatment on norepinephrine induced cardiovascular responses was studied. Injection of norepinephrine (0.5, 1.0 and 2.0 μg/kg, i.v.) alone produced a dose-dependent pressor effect, while heart rate was decreased. DCLHb pretreatment produced a significant ($F(2,18) = 64.87$; $P < 0.0005$) potentiation of the pressor effect of norepinephrine (fig. 3). In order to determine the specificity of DCLHb-induced potentiation of norepinephrine effects, pretreatment was done with phenoxybenzamine (5 mg/kg, i.v.). The potentiation of the norepinephrine induced pressor effect by DCLHb was completely blocked ($F(2,18) = 57.56$; $P < 0.0005$) by phenoxybenzamine (fig. 3). Since phenoxybenzamine is a specific α-adrenoceptor antagonist, the specificity of the potentiated norepinephrine response by DCLHb was established. Heart rate responses induced by norepinephrine were not significantly affected by DCLHb pretreatment ($F(2,18) = 2.75$; $P = 2.637$) except at the highest dose of norepinephrine, ($F(2,18) = 13.64$; $P = 0.003$) which was blocked by phenoxybenzamine (fig. 3). The basal systolic blood pressure was 110 ± 6 mmHg before and 146 ± 4 mmHg after DCLHb administration.

Effect of DCLHb on phenylephrine induced cardiovascular responses

In order to determine the subtypes of α-adrenoceptors involved, the effect of DCLHb pretreatment on phenylephrine, a specific α_1-adrenoceptor agonist, induced cardiovascular responses was studied. Phenylephrine (5, 10 and 20 μg/kg, i.v.) produced a dose-dependent pressor effect with a reflex decrease in heart rate. DCLHb (400 mg/kg, i.v.) pretreatment significantly ($F(2,20) = 91.63$; $P < 0.0005$) potentiated the pressor effect of phenylephrine at all the doses studied. Significant ($F(2,20) = 46.68$; $P < 0.0005$) potentiation of the phenylephrine induced decrease in heart rate was also produced by DCLHb (fig. 4). In order to determine the specificity, pretreatment was done with a specific α_1-adrenoceptor antagonist, prazosin. The potentiation of the pressor response of phenylephrine (5, 10 and 20 μg/kg, i.v.) by DCLHb was

completely blocked by prazosin (1 mg/kg, i.v.) ($F(2,20) = 45.88$; $P < 0.0005$). The potentiation of the phenylephrine induced decrease in heart rate by DCLHb was also significantly ($F(2,20) = 31.81$; $P < 0.0005$) blocked by prazosin (fig. 4). This clearly indicated that DCLHb potentiated the α_1-adrenoceptor mediated responses. The basal systolic blood pressure was 127 ± 5 mmHg before and 159 ± 7 mmHg after DCLHb administration.

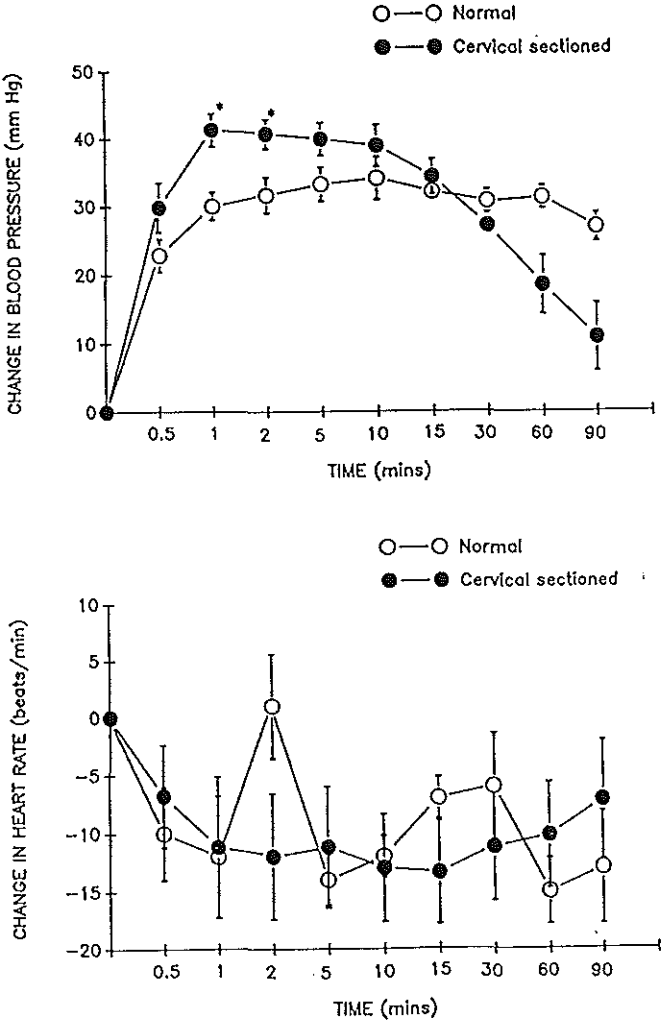

Fig. 1 The effect of DCLHb (400 mg/kg, i.v.) on mean arterial blood pressure (mmHg) and heart rate (beats/min) in normal (intact) (N=5) and cervical sectioned (N=5) rats. *indicates significant difference as compared to normal rats.

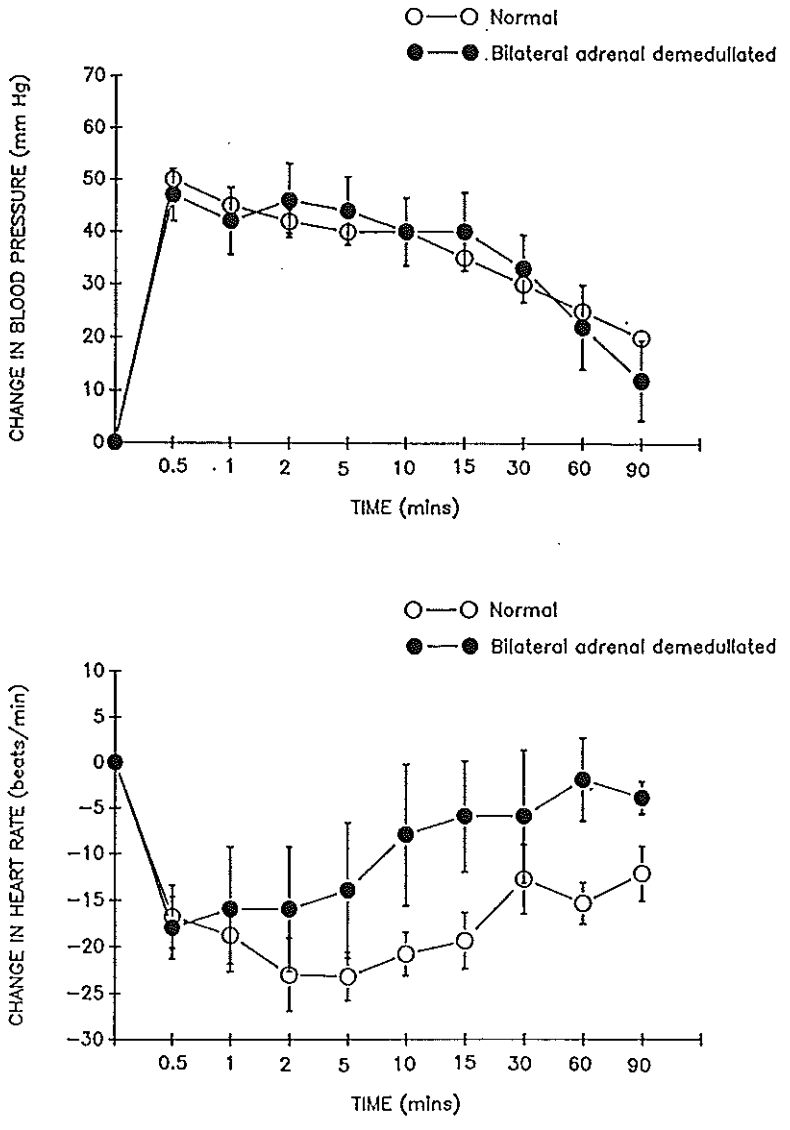

Fig. 2 The effect of DCLHb (400 mg/kg, i.v.) on mean arterial blood pressure (mmHg) and heart rate (beats/min) in normal (intact) (N=5) and bilateral adrenal demedullated (N=5) rats.

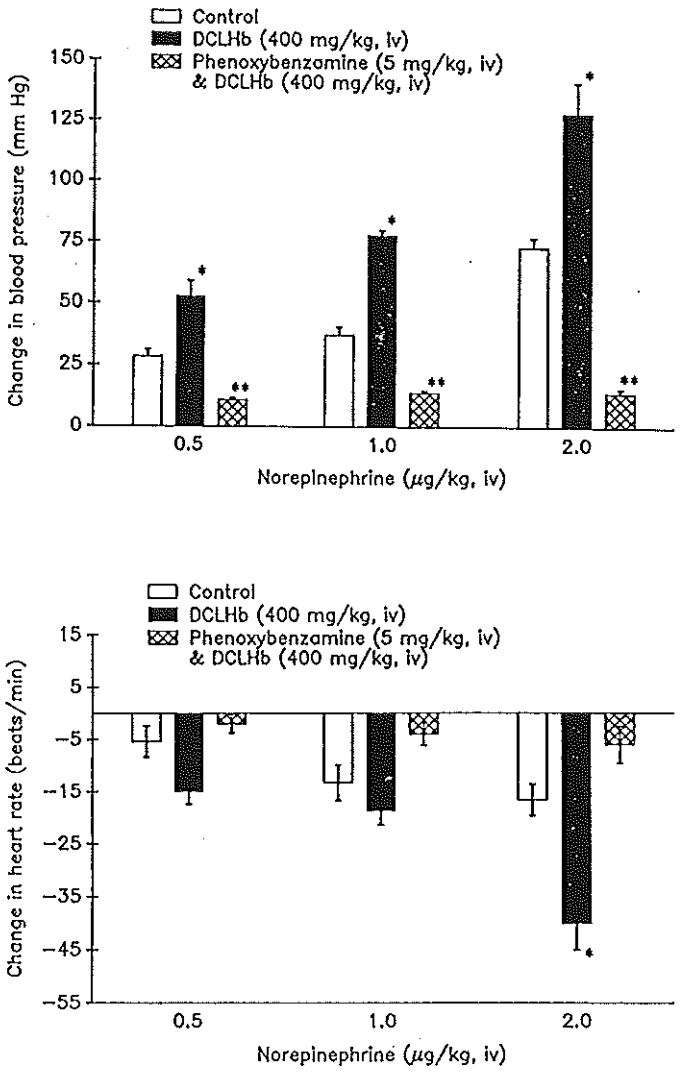

Fig. 3 The effect of DCLHb on the potentiation of norepinephrine (0.5, 1.0 and 2.0 μg/kg, i.v.) induced increase in blood pressure and heart rate. Norepinephrine was given (1) alone in normal rats (control) (N=12), (2) 15 min after treatment with DCLHb (400 mg/kg, i.v.) (N=4) and (3) 15 min after treatment with DCLHb (400 mg/kg, i.v.) and phenoxybenzamine (POB) (5 mg/kg, i.v.) (N=5). *indicates significant difference as compared to control group; #indicates significant difference as compared to DCLHb group.

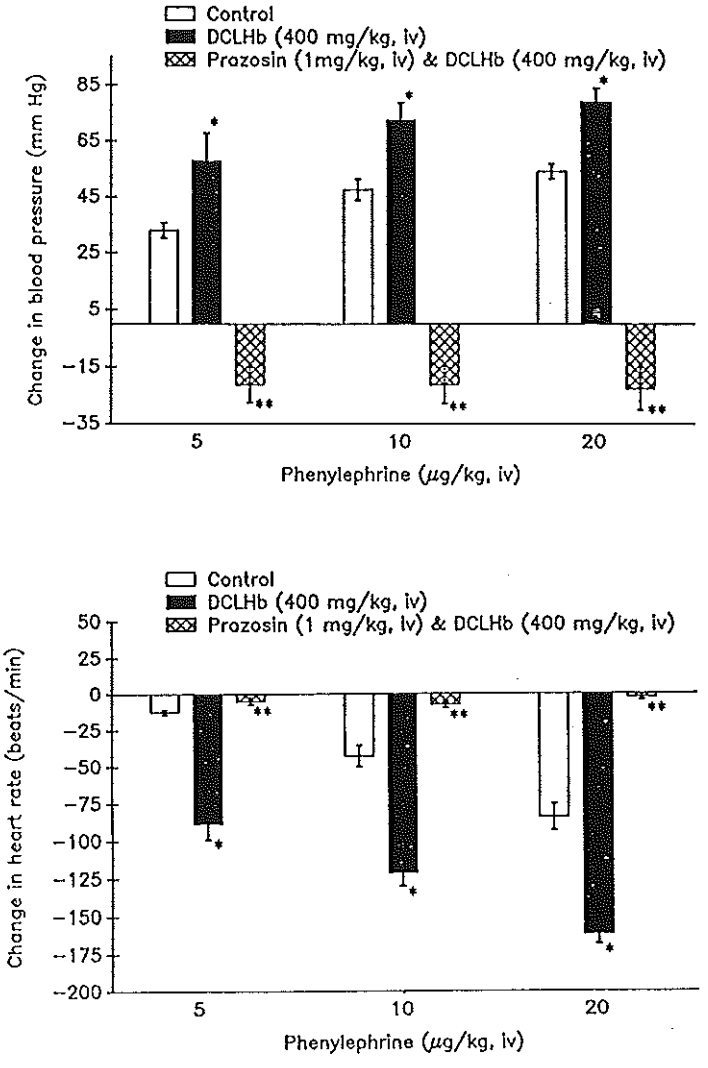

Fig. 4 The effect of DCLHb on the potentiation of phenylephrine (5.0, 10.0 and 20.0 μg/kg, i.v.) induced increase in blood pressure and heart rate. Phenylephrine was given (1) alone in normal rats (control) (N=12), (2) 15 min after treatment with DCLHb (400 mg/kg, i.v.) (N=8) and (3) 15 min after treatment with DCLHb (400 mg/kg, i.v.) and prazosin (PRZ) (1 mg/kg, i.v.) (N=4). *indicates significant difference as compared to control group; #indicates significant difference as compared to DCLHb group.

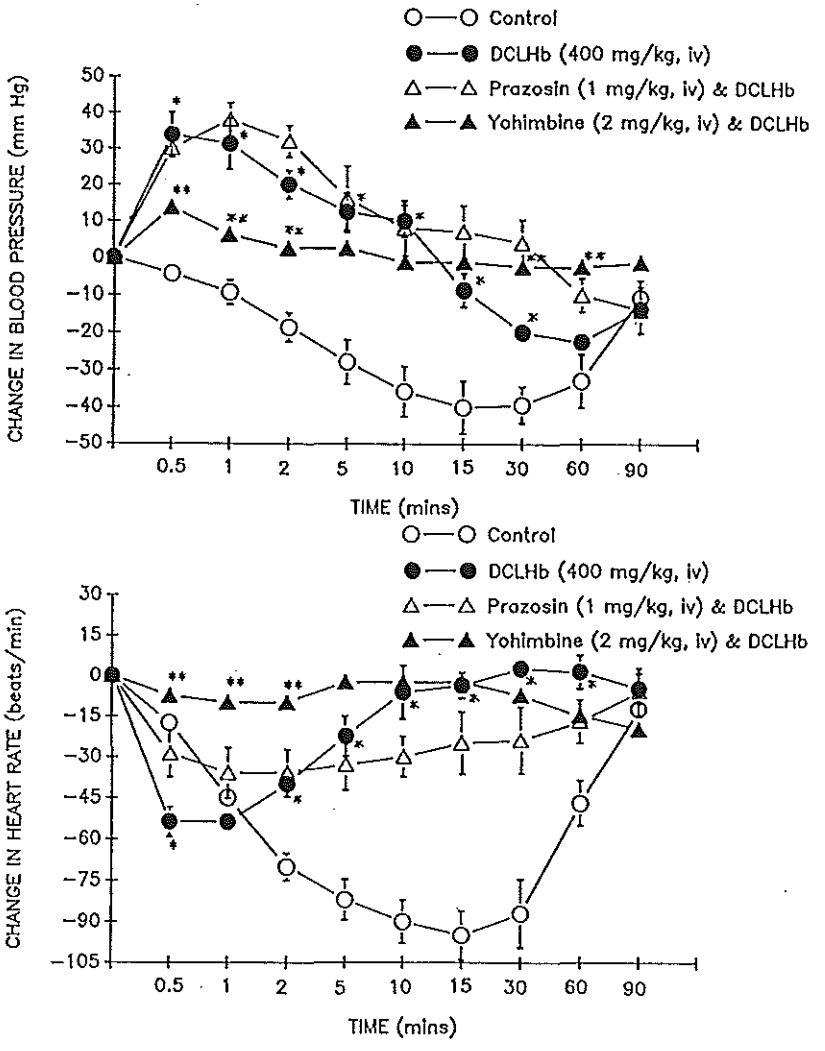

Fig. 5 The effect of DCLHb on blood pressure and heart rate changes induced by clonidine (75 μg/kg, i.v.) in rats. Clonidine was given (1) alone in normal rats (control) (N=4), (2) 15 min after treatment with DCLHb (400 mg/kg, i.v.) (N=4), (3) 15 min after treatment with DCLHb (400 mg/kg, i.v.) and prazosin (PRZ) (1 mg/kg, i.v.) (N=5) and (4) 15 min after treatment with DCLHb (400 mg/kg, i.v.) and yohimbine (YMB) (2 mg/kg, i.v.) (N=4). *indicates significant difference as compared to control group; #indicates significant difference as compared to DCLHb group.

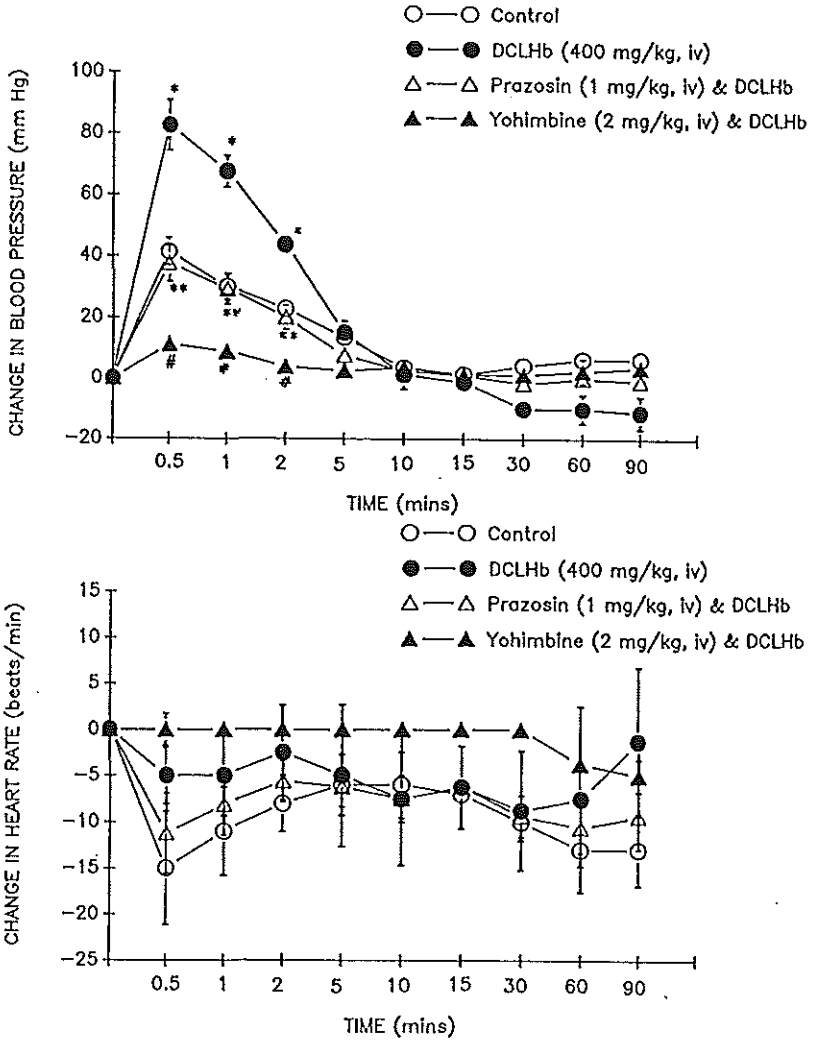

Fig. 6 The effect of DCLHb on blood pressure and heart rate changes induced by clonidine (25 μg/kg, i.v.) in cervical sectioned rats. Clonidine was given (1) alone in sectioned rats (control) (N=7), (2) 15 min after treatment with DCLHb (400 mg/kg, i.v.) (N=4), (3) 15 min after treatment with DCLHb (400 mg/kg, i.v.) and prazosin (PRZ) (1 mg/kg, i.v.) (N=6) and (4) 15 min after treatment with DCLHb (400 mg/kg, i.v.) and yohimbine (YMB) (2 mg/kg, i.v.) (N=4). *indicates significant difference as compared to control group; #indicates significant difference as compared to DCLHb group; + indicates significant difference as compared to prazosin + DCLHb group.

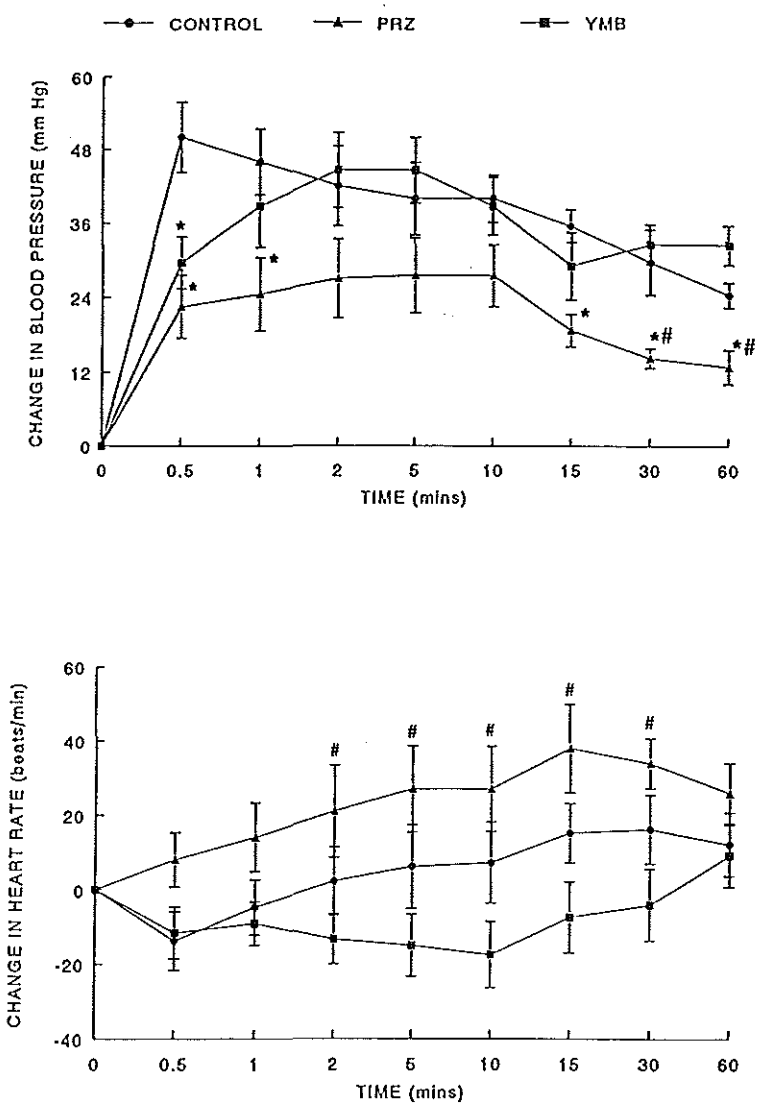

Fig. 7 The effect of DCLHb on the change in blood pressure and heart rate in control (untreated) (N=5), yohimbine (YMB) (N=6) and prazosin (PRZ) (N=5) pretreated rats. Yohimbine (2 mg/kg, i.v.) or prazosin (1 mg/kg, i.v.) treatment was done 15 min prior to the administration of DCLHb. *indicates significant difference as compared to control group; #indicates significant difference as compared to yohimbine treated group.

Effect of DCLHb on clonidine induced cardiovascular effects in normal rats

Clonidine is a centrally acting hypotensive agent, but can produce a hypertensive response by acting on peripheral vascular α-adrenoceptors. In the present study the effect of DCLHb on clonidine-induced cardiovascular responses was investigated. Clonidine (75 μg/kg, i.v.) injection alone produced a fall in blood pressure and a decrease in heart rate. DCLHb (400 mg/kg, i.v.) pretreatment significantly ($F(3,16) = 35.02$; $P < 0.0005$) blocked the hypotensive effect of clonidine. Instead of a fall in blood pressure, a rise in blood pressure was observed with clonidine (75 μg/kg, i.v.) in DCLHb pretreated rats. The decrease in heart rate was also attenuated ($F(3,13) = 9.98$; $P = 0.01$) by DCLHb pretreatment (fig. 5). In order to determine the role of subtypes of α-adrenoceptors, animals were pretreated with a specific α_1-adrenoceptor antagonist, prazosin, or with a specific α_2-adrenoceptor antagonist, yohimbine. When rats were pretreated with prazosin (1 mg/kg, i.v.), the potentiation of the hypertensive effect of clonidine by DCLHb was not affected ($P = 0.886$). However, when rats were pretreated with yohimbine (2 mg/kg, i.v.), the potentiation of the clonidine-induced hypertensive effect by DCLHb was significantly blocked ($P = 0.008$) (fig. 5). The basal systolic blood pressure was 128 ± 7 mmHg before and 148 ± 3 mmHg after DCLHb administration.

Effect of DCLHb on clonidine induced cardiovascular effects in cervical sectioned rats

In cervical sectioned rats the central hypotensive effect of clonidine was not observed. Even a low dose of clonidine (25 μg/kg, i.v.) produced some pressor effect, but there was no ($F(3,18) = 1.657$; $P = 0.717$) effect on heart rate. DCLHb (400 mg/kg, i.v.) pretreatment significantly ($F(3,17) = 20.832$; $P = 0.001$) potentiated the pressor effect of clonidine, while heart rate was not affected (fig. 6). In order to determine the role of subtypes of α-adrenoceptors, animals were pretreated with a specific α_1-adrenoceptor antagonist, prazosin, or with a specific α_2-adrenoceptor antagonist, yohimbine. After prazosin (1 mg/kg, i.v.) pretreatment, the potentiation of the pressor effect of clonidine by DCLHb was significantly ($F(3,17) = 20.832$; $P = 0.001$) blocked. However, yohimbine (2 mg/kg, i.v.) pretreatment not only blocked the potentiation of the pressor effect of clonidine by DCLHb ($P < 0.0005$) but also the pressor effect of clonidine alone (fig. 6).

Effect of α adrenoceptor blockers on DCLHb induced cardiovascular responses

DCLHb (400 mg/kg, i.v.) produced a significant increase in blood pressure, but heart rate was not altered. Yohimbine (2 mg/kg, i.v.) pretreatment 15 mins before the administration of DCLHb significantly ($F(2,13) = 7.82$; $P = 0.016$) attenuated the pressor effect of DCLHb in the initial stages. Prazosin (1 mg/kg, i.v.) pretreatment 15 mins before the administration of DCLHb significantly attenuated ($F(2,13) = 7.82$; $P = 0.005$) its pressor effect (fig. 7). The attenuation of the pressor effect of DCLHb was more significant by prazosin as compared to yohimbine ($F(2,13) = 13.87$; $P < 0.0005$). Heart rate responses were not affected significantly (fig. 7). The maximal increase in blood pressure by DCLHb was less in both yohimbine and prazosin

pretreated rats as compared to normal rats, due to the hypotensive effects of yohimbine and prazosin. The maximal increase in blood pressure by DCLHb in prazosin pretreated rats was not more than the basal blood pressure observed before the administration of prazosin.

5.4 Discussion

The hemodynamic response to blood replacement with stroma-free haemoglobin differs from that observed with other fluids (Vogel *et al.*, 1986) and has been attributed to improved systemic oxygen delivery (Hauser *et al.*, 1982). However, the vascular response to stroma-free haemoglobin is believed to be independent of oxygen affinity (Nees *et al.*, 1978). Although most stroma-free haemoglobin solutions have been reported to produce a pressor effect, (Przybelski *et al.*, 1991; Kida *et al.*, 1991; Messmer *et al.*, 1977; Rabinovici *et al.*, 1989) the mechanisms responsible for the pressor effect are not known. This is the first study to investigate the role of adrenergic mechanisms in the pressor effect of DCLHb.

The present studies show that DCLHb produces a pressor effect in rats. When given intravenously in cervical sectioned rats, DCLHb produced an increase in blood pressure greater than that observed in normal rats. However, the duration of the pressor effect in cervical sectioned rats was less than normal rats. Since DCLHb produced its pressor effect when the influence of the CNS in blood pressure regulation was removed, it is clear that the pressor effect of DCLHb was mediated through the peripheral vascular system rather than through the CNS.

It could be possible that DCLHb releases catecholamines or other pressor substances from the adrenal medulla. In order to investigate the role of the adrenal medulla in the pressor effect of DCLHb, experiments were performed in bilateral adrenal demedullated rats. DCLHb produced a similar pressor effect in bilateral adrenal demedullated rats and normal rats. It is thus clear that DCLHb does not produce a pressor response by releasing catecholamines or other pressor substances from the adrenal medulla.

The above studies indicate that DCLHb produces a pressor effect through the peripheral vascular system and that the CNS and adrenal medullary systems are not involved. It is possible that DCLHb alters the vascular sensitivity of α-adrenoceptors which, when stimulated, will produce vasoconstriction and an increase in blood pressure. In order to test this hypothesis, the effects of DCLHb pretreatment on norepinephrine induced blood pressure and heart rate responses were studied. DCLHb significantly potentiated the pressor response to norepinephrine but it did not affect the heart rate response. Since heart rate responses are mainly mediated through the β-adrenoceptors or through reflex action, while the pressor responses are mediated through the α-adrenoceptors (Hoffman and Lefkowitz, 1990a), it appears that DCLHb does not affect the β-adrenoceptors but significantly potentiates the α-adrenoceptor mediated responses.

The specificity of the potentiation was confirmed by using phenoxybenzamine, a specific α-adrenoceptor antagonist (Hoffman and Lefkowitz, 1990b), which completely blocked the DCLHb induced potentiation of norepinephrine responses. Further studies were carried out to determine the subtypes of α-adrenoceptors sensitized by DCLHb. Phenylephrine, a specific α_1-adrenoceptor agonist (Hoffman and Lefkowitz, 1990a), produced a dose dependent pressor effect and a reflex decrease in heart rate. DCLHb significantly potentiated the pressor and heart rate effects of phenylephrine. The potentiation of α_1-adrenoceptor mediated responses by DCLHb was blocked by a specific antagonist, prazosin, acting on the α_1-adrenoceptors (Hoffman and Lefkowitz, 1990a). Since phenylephrine acts directly on α_1-adrenoceptors, it appears that DCLHb sensitizes the α-adrenoceptors directly rather than affecting the catecholamine release and uptake processes.

Clonidine, an α_2-adrenoceptor agonist, produces a fall in blood pressure by acting on the central α-adrenoceptors (Kobinger, 1978; Schmitt, 1971; Langer, 1981) and a rise in blood pressure by stimulating the peripheral vascular α-adrenoceptors (Langer, 1981; Frisk-Holmberg, 1984). DCLHb produced a marked potentiation of the pressor response to clonidine, supporting the earlier observations that DCLHb increases the sensitivity of peripheral vascular receptors. The pressor response to clonidine was potentiated to the extent that the central hypotensive effect of clonidine was not observed in DCLHb pretreated rats.

In order to determine the specificity of the DCLHb induced potentiation of the pressor effect of clonidine, specific α_1- and α_2-adrenoceptor antagonists were used. Pretreatment with prazosin, an α_1-adrenoceptor antagonist, did not attenuate the DCLHb-induced potentiation of the pressor effect of clonidine in intact rats. However, pretreatment with yohimbine, an α_2-adrenoceptor antagonist (Hoffman and Lefkowitz, 1990b), completely blocked the DCLHb induced potentiation of the clonidine induced pressor response in intact rats. This indicated that DCLHb besides α_1-adrenoceptors also sensitized α_2-adrenoceptors. Further studies were performed in cervical sectioned rats, where the central hypotensive effect of clonidine was not present. DCLHb markedly potentiated the pressor effect of clonidine in cervical sectioned rats. The potentiation of the clonidine induced pressor response by DCLHb could be attenuated by prazosin. Yohimbine completely blocked the pressor effect of clonidine in cervical sectioned rats. Therefore, it appears that both α_1- and α_2-adrenoceptors in the peripheral vascular system are sensitized by DCLHb.

In order to further confirm that pressor effect of DCLHb is mediated through the α_1- and α_2-adrenoceptors, the effect of pretreatment of specific α adrenoceptor antagonists on the pressor response of DCLHb was studied. DCLHb produced a significant increase in the blood pressure in rats. Yohimbine attenuated the pressor effect of DCLHb in the initial stages. The maximal increase in the blood pressure by DCLHb was significantly reduced by yohimbine pretreatment.

Prazosin significantly blocked the pressor effect of DCLHb and there was only a minimal increase in blood pressure by DCLHb in prazosin pretreated rats.

It was found that pyridoxalated haemoglobin polyoxyethylene conjugate (PHP) does not affect the vasodilator actions of acetylcholine and nitroglycerine (Kida *et al.*, 1991). The constrictor responses to angiotensin II and 5-HT after exchange transfusion with PHP were not found to be altered (Kida *et al.*, 1991). However, the responses to norepinephrine were significantly augmented in PHP transfused rats (Kida *et al.*, 1991). These findings support our observations that DCLHb increases the sensitivity of vascular α adrenoceptors. The mechanisms involved in increasing the sensitivity of vascular α adrenoceptors are not known. It could be due to altered metabolism. Metabolic signals such as H^+ have been shown to alter the sensitivity of arteriolar adrenergic constriction (McGillivray-Anderson and Faber, 1991). It could be possible that DCLHb sensitizes the α adrenoceptors either directly or through endothelin mechanisms. Phosphoramidon, an inhibitor of pro-endothelin conversion to endothelin, attenuated the elevation of blood pressure by DCLHb in rats indicating that the pressor effect of DCLHb might be mediated through endothelin mechanisms (Schultz *et al.*, 1993). L-arginine, the substrate for NO and nitroglycerine, a NO donor, significantly reduced the pressor effect of DCLHb, when infused in rats 15 min after the administration of DCLHb (Schultz *et al.*, 1993). The potentiation of adrenergic responses by a vasoconstrictor peptide, endothelin has been observed (Gulati and Srimal, 1993). DCLHb could be sensitizing peripheral vascular α adrenoceptors through endothelin mechanism.

It can be concluded that: (1) The pressor effect of DCLHb is not likely to be mediated through CNS mechanisms, but appears to be mediated through the peripheral vascular system. (2) The pressor effect of DCLHb is not mediated through the release of catecholamines or other pressor substances from the adrenal medulla. (3) DCLHb potentiates the pressor responses to norepinephrine, phenylephrine and clonidine possibly due to the increased sensitivity of peripheral vascular α-adrenoceptors. (4) The pressor effect of DCLHb could be attenuated by yohimbine and more significantly by prazosin pretreatment.

5.5 Acknowledgments

This work was support by a grant from Blood Substitute Group, Baxter Healthcare Corp. to Anil Gulati.

Chapter 6

Prazosin blocks the pressor but not the regional circulatory effects of diaspirin crosslinked haemoglobin

Summary

Diaspirin crosslinked haemoglobin (DCLHb™) (400 mg/kg, i.v.) produces an increase in blood pressure and blood flow to the heart, spleen, stomach, small intestine, skin, mesentery and pancreas when administered to rats. The present study was conducted to determine (1) whether prazosin, an α_1-adrenergic antagonist, can block the pressor effect of DCLHb and (2) the effect of prazosin pretreatment on regional circulatory changes induced by DCLHb in rats. DCLHb (400 mg/kg, i.v.) produced an increase in blood pressure (64%), cardiac output (20%) and total peripheral resistance (65%) when administered to control rats. Infusion of DCLHb in prazosin (1 mg/kg, i.v.) treated rats did not show any significant pressor effect, but reversed the hypotensive effect of prazosin. Cardiac output and stroke volume were significantly increased and total peripheral resistance decreased in prazosin treated rats as compared to control (untreated) rats. DCLHb significantly increased blood flow to the heart, gastrointestinal tract, portal system (spleen), and skin of control rats. Blood flow to the brain, kidneys, and musculo-skeletal system was not altered following the infusion of DCLHb in control rats. Infusion of DCLHb in prazosin treated rats produced a significant increase in blood flow to the brain, heart, kidneys, gastrointestinal tract, portal system, skin and musculoskeletal system. In summary, prazosin pretreatment blocked the pressor effect of DCLHb, however, blood flow to the heart, brain, gastrointestinal tract, portal system, kidneys, skin and musculoskeletal system was increased by DCLHb. It is concluded that blood flow to most of the organs is increased by DCLHb but the pressor effect of DCLHb is blocked by prazosin pretreatment.

6.1 Introduction

Several investigators have stressed the advantages of haemoglobin solutions as compared to other resuscitating solutions (Bonhard, 1975; Kaplan and Murphy, 1975; Moss *et al.*, 1976; DeVenuto and Zegna, 1978). Haemoglobin based blood substitutes are therefore being developed as promising resuscitative solutions. Diaspirin crosslinked haemoglobin (DCLHb) is a blood substitute derived from the haemoglobin of outdated erythrocytes and produced by crosslinking molecular haemoglobin between the α-subunits by a reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process includes heat pasteurization of the solution, as described earlier (Estep *et al.*, 1989b; Estep *et al.*, 1989a). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). The crosslinking of the α subunits affords the haemoglobin a favorable oxygen dissociation curve (Snyder *et al.*, 1987; Vandegriff *et al.*, 1989).

DCLHb has also been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion in rats (Cole *et al.*, 1992). Partial or complete exchange transfusion with DCLHb did not significantly affect the cardiac and renal functions in swine (Hess *et al.*, 1989). DCLHb has been found to be a promising resuscitative fluid following hemorrhage (Przybelski *et al.*, 1990). In hemorrhaged rats DCLHb (10 ml/kg of 14 %) was as efficacious as whole blood in the restoration of cardiovascular and tissue oxygenation parameters (Przybelski *et al.*, 1991).

DCLHb increases the mean arterial pressure when administered to hemorrhaged (Przybelski *et al.*, 1991) or normal rats (Hamilton *et al.*, 1992). The pressor effect of DCLHb (125-4000 mg/kg, iv) was found to be self limiting (Hamilton *et al.*, 1992). Other haemoglobin solutions have also been reported to produce a rapid and sustained increase in mean arterial pressure (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). DCLHb has been shown to produce significant changes in regional blood circulation (Sharma *et al.*, 1994). Although, DCLHb has been found to increase the pressor responses to α-adrenoceptor agonists (Gulati and Rebello, 1994), the regional circulatory effect of DCLHb was found to be different from that of norepinephrine (Sharma and Gulati, 1994). Prazosin, an α_1-adrenoceptor antagonist, is an effective antihypertensive agent (Weiner, 1980). The present study was performed with the aim of determining whether prazosin can attenuate the pressor effect of DCLHb without compromising the increase in regional blood flow.

6.2 Materials and Methods

Determination of systemic hemodynamics and regional circulation

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 300-350 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure. In order to keep the blood pO_2, pCO_2 and pH constant and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683. The arterial pO_2 and pCO_2 were kept within 85-120 mmHg and 35-50 mmHg range, respectively. The carotid artery of the right side was exposed and a PE 50 cannula guided through the common carotid artery to the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P23 DC pressure transducer. When the

cannula reached the ventricle the diastolic pressure dropped to zero. The femoral artery of the right side was cannulated and connected to a withdrawal pump (Harvard Model 22).

At each measurement, a suspension of approximately 200,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA) in 0.2 ml saline were injected into the left ventricle after thoroughly mixing and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all tissues and organs were dissected out, weighed and placed in vials containing 10% formalin. The following tissues were studied: lungs, heart, liver, stomach, small intestine, caecum, large intestine, mesentery and pancreas, spleen, left kidney, right kidney, left cerebral hemisphere, right cerebral hemisphere, midbrain, cerebellum, brain stem, skin and the rest of the body consisting of muscles and bones. The radioactivity in standards, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output, (2) stroke volume, (3) total peripheral resistance, (4) regional blood flow and (5) regional vascular resistance. Results were calculated using the programs described earlier (Saxena *et al.*, 1980). Infusion of saline did not affect the systemic hemodynamics and regional circulation significantly. The effect of DCLHb (400 mg/kg, i.v.) on systemic hemodynamics and regional circulation was studied. DCLHb was infused as a 10 % solution in volume of 4 ml/kg intravenously. The dose of DCLHb was selected on the basis of studies conducted previously (Hamilton *et al.*, 1992; Sharma *et al.*, 1994; Gulati and Rebello, 1994), which demonstrated that the infusion of this dose of DCLHb resulted in a near maximal pressor response.

Statistics

All data are presented as the mean values ± 1 SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Duncan's test. A level of $P < 0.05$ was considered significant.

6.3 Results

Effect of prazosin pretreatment on DCLHb induced changes in systemic hemodynamics

DCLHb (400 mg/kg, i.v.) produced an increase ($F(3,28) = 10.78$; $P < 0.0001$) in blood pressure of control rats. This increase in blood pressure was significantly ($F(7,44) = 13.63$; $P < 0.0001$) attenuated in prazosin treated rats (Figure 1). Prazosin (1 mg/kg, iv) decreased blood

pressure from 83.6 mmHg to 64.5 mmHg. Following administration of DCLHb the maximal increase in blood pressure observed was 81.5 mmHg (Figure 1). Heart rate was not significantly affected by the administration of DCLHb in control or prazosin treated rats (Figure 2). Infusion of DCLHb or prazosin alone did not produce a significant change in the cardiac output of control rats. However, infusion of DCLHb in prazosin treated rats produced a significant increase in cardiac output ($F(4,20) = 11.03$; $P = 0.0001$) 15, 30 and 60 min after DCLHb administration (Figure 3). DCLHb did affect stroke volume in control rats, while administration of prazosin produced no significant effect on stroke volume. However, when DCLHb was administered in prazosin treated rats, the stroke volume was significantly ($F(4,20) = 11.42$; $P = 0.0001$) increased (Figure 4). The infusion of DCLHb produced a significant ($F(3,28) = 6.53$; $P = 0.0001$) increase in total peripheral resistance of control rats 30 and 60 min after administration. Prazosin did not produce any significant effect on total peripheral resistance. When DCLHb was administered in prazosin treated rats, total peripheral resistance significantly ($F(7,44) = 11.58$; $P < 0.0001$) decreased as compared to control rats (Figure 5).

Effect of prazosin pretreatment on DCLHb induced changes in regional blood flow

Infusion of DCLHb produced a significant ($P < 0.05$) increase in blood flow to the heart, gastrointestinal tract, portal system and skin. Blood flow to the brain, kidneys and musculo-skeletal system was not affected in control rats. The increase in blood flow to most organs occurred 15 and 30 min after administration of DCLHb. Blood flow tended to return to baseline by 60 min post-DCLHb administration (Figure 6). Prazosin treatment did not alter the blood flow in any region. When administered to prazosin treated rats, DCLHb produced a significant ($P < 0.05$) increase in blood flow to the heart, brain, gastrointestinal tract, portal system, kidneys, skin and musculoskeletal system (Figure 6).

Blood flow was also determined in different brain regions. Infusion of DCLHb to control rats did not affect blood flow to the left and right cerebral hemispheres, diencephalon, cerebellum or brain stem. Prazosin per se did not significantly alter the blood flow to any region of the brain. When administered to prazosin treated rats, DCLHb produced a significant increase in blood flow to the left and right cerebral hemispheres, diencephalon, cerebellum and brain stem (Figure 7).

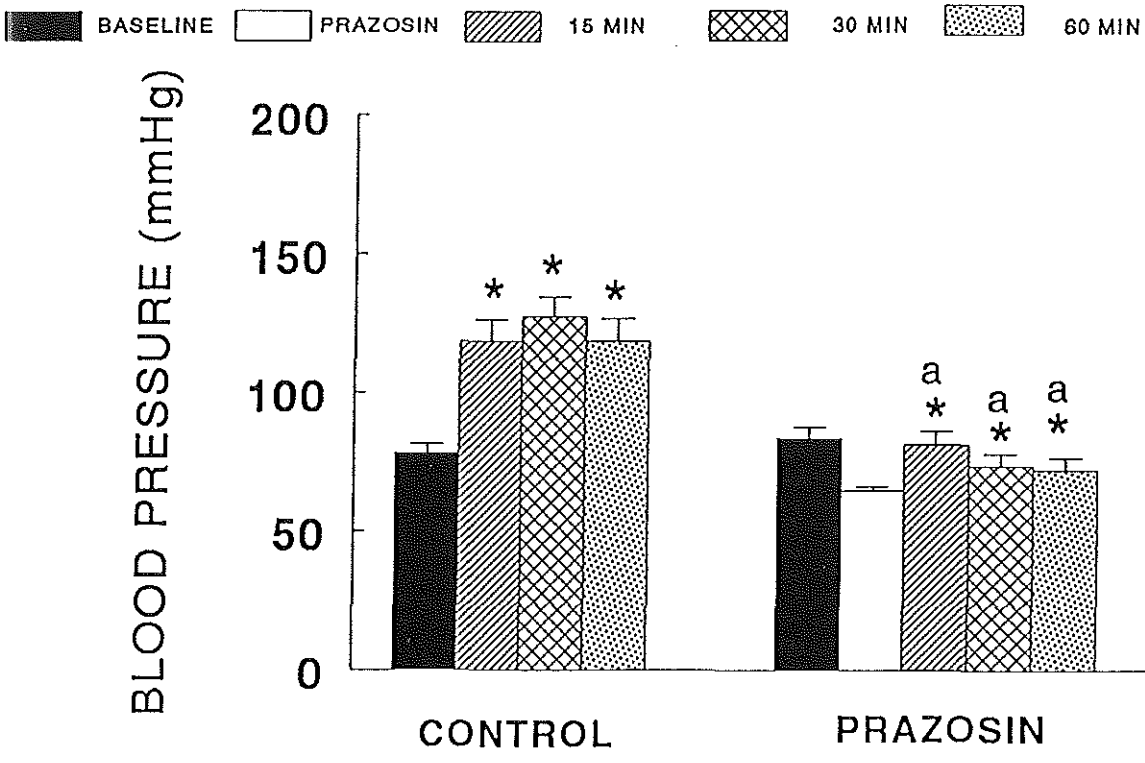

Fig. 1 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the mean arterial blood pressure (mmHg) of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb. *Indicates significant difference as compared to baseline (control) or prazosin basal values (15 min after prazosin treatment); [a]indicates significant difference as compared to control (untreated rats).

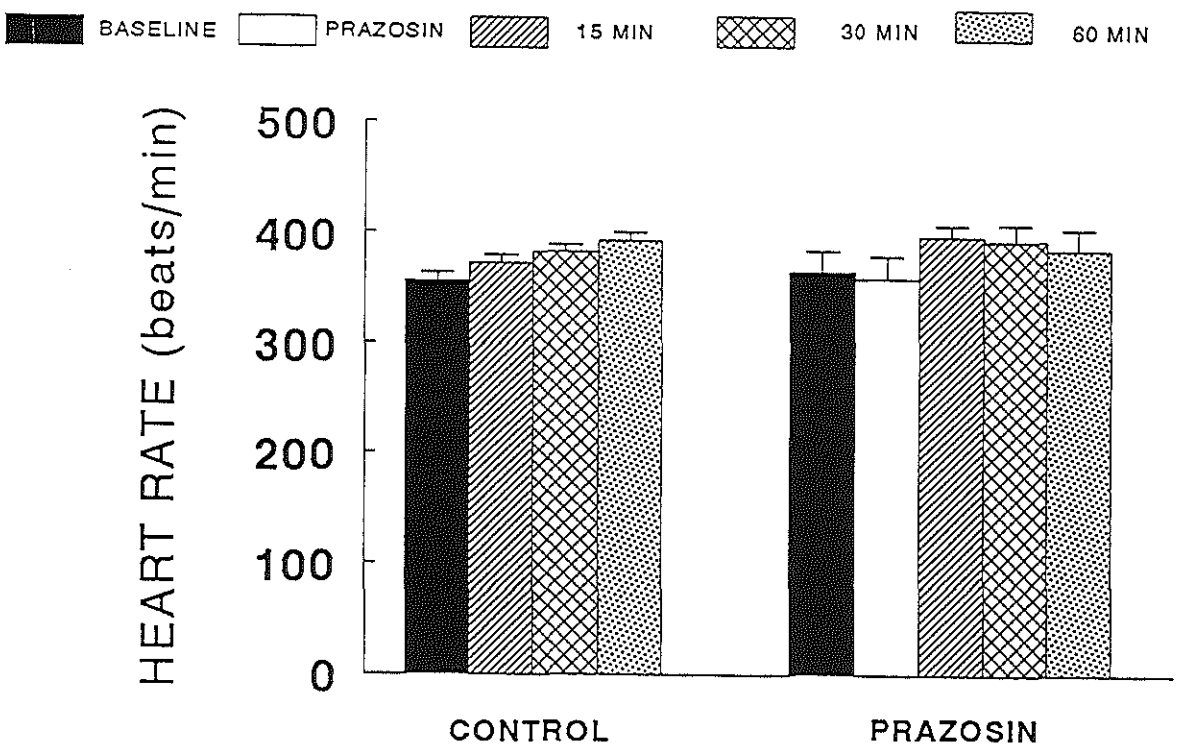

Fig. 2 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the heart rate (beats/min) of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb.

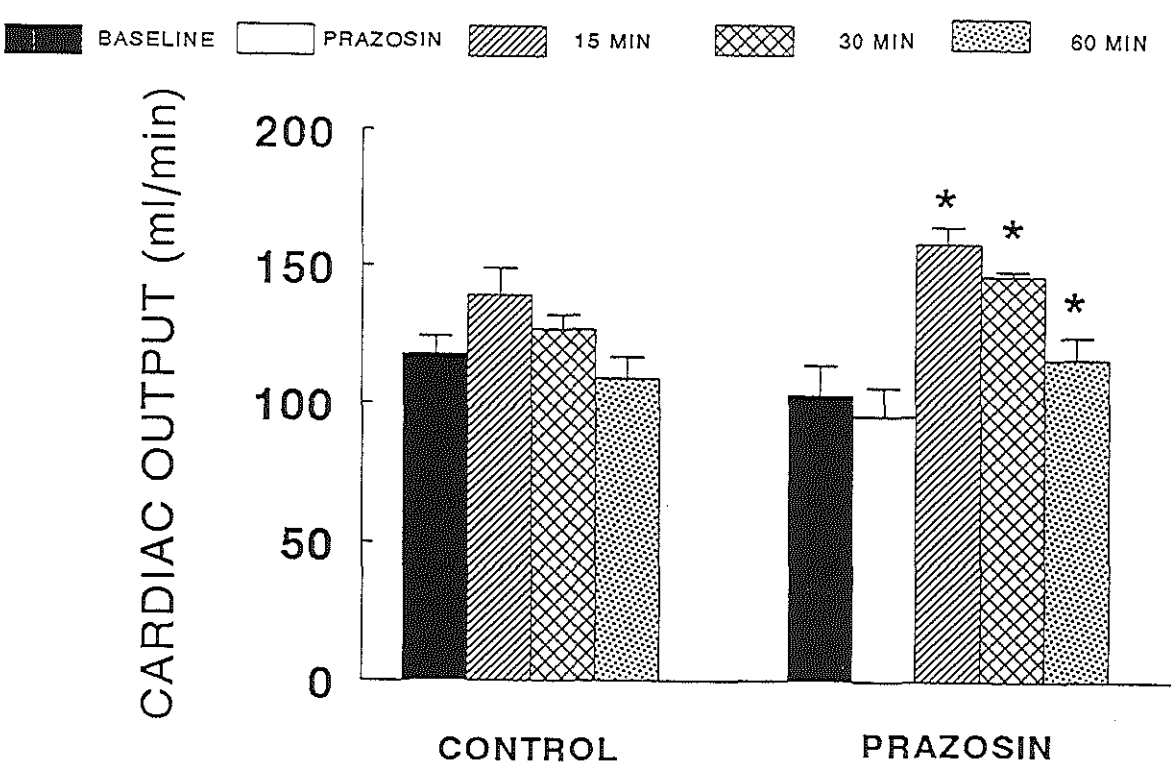

Fig. 3 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the cardiac output (ml/min) of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb. *Indicates significant difference as compared to baseline (control) or prazosin basal values (15 min after prazosin treatment).

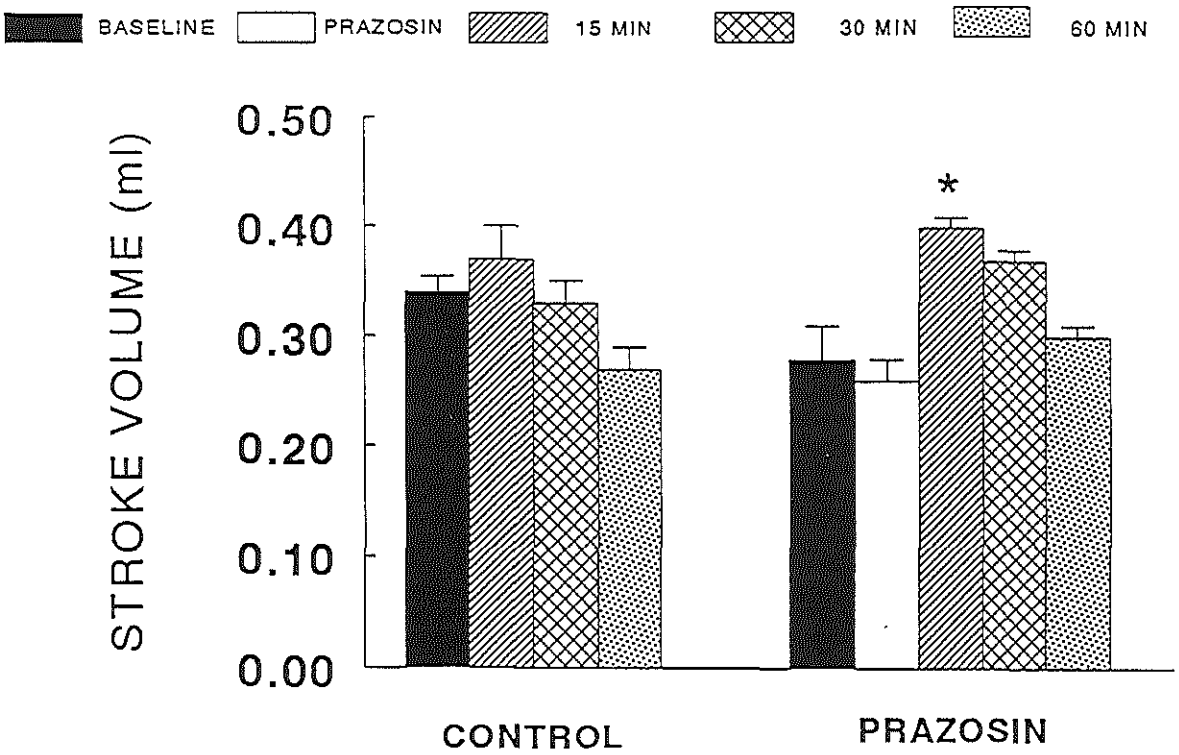

Fig. 4 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the stroke volume (ml) of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb. *Indicates significant difference as compared to baseline (control) or prazosin basal values (15 min after prazosin treatment).

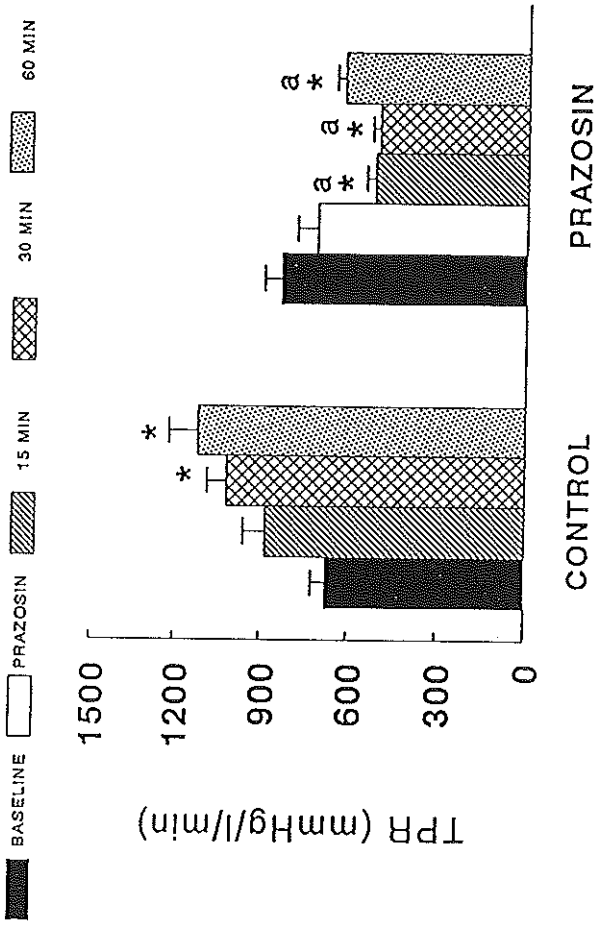

Fig. 5 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the total peripheral resistance (mmHg/l/min) of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb.
*Indicates significant difference as compared to baseline (control) or prazosin basal values (15 min after prazosin treatment);
ªindicates significant difference as compared to control (untreated rats).

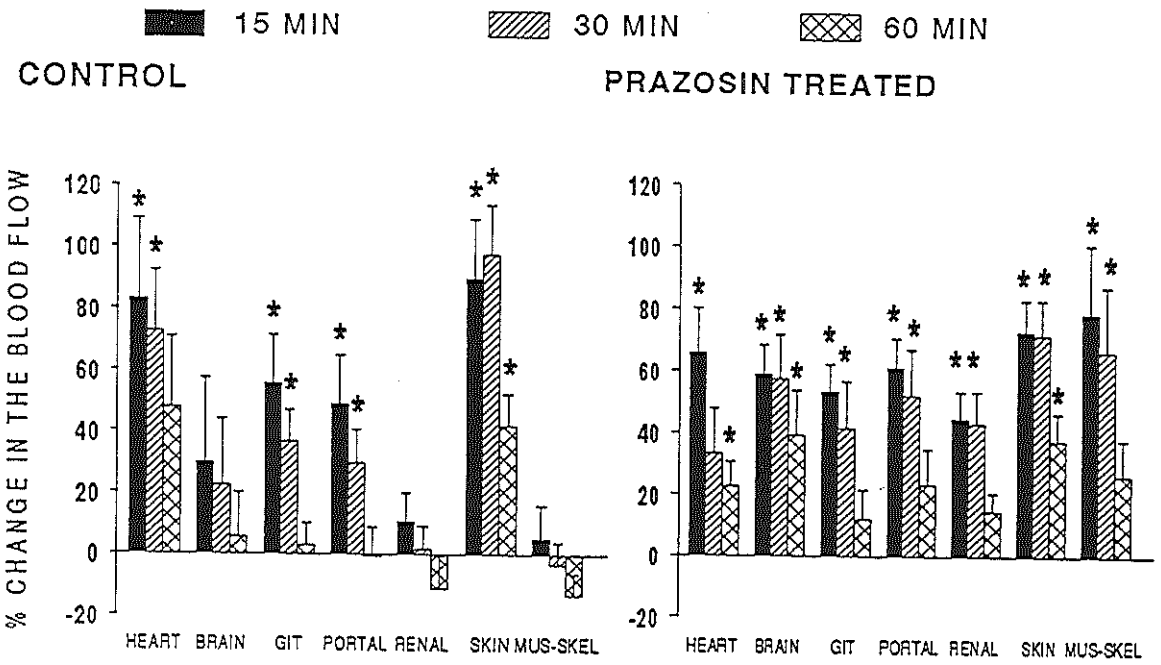

Fig. 6 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the percentage change in the blood flow to the heart, brain, gastrointestinal tract (GIT), portal system, renal, skin and musculoskeletal system of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb. *Indicates significant difference as compared to baseline (control) or prazosin basal values (15 min after prazosin treatment).

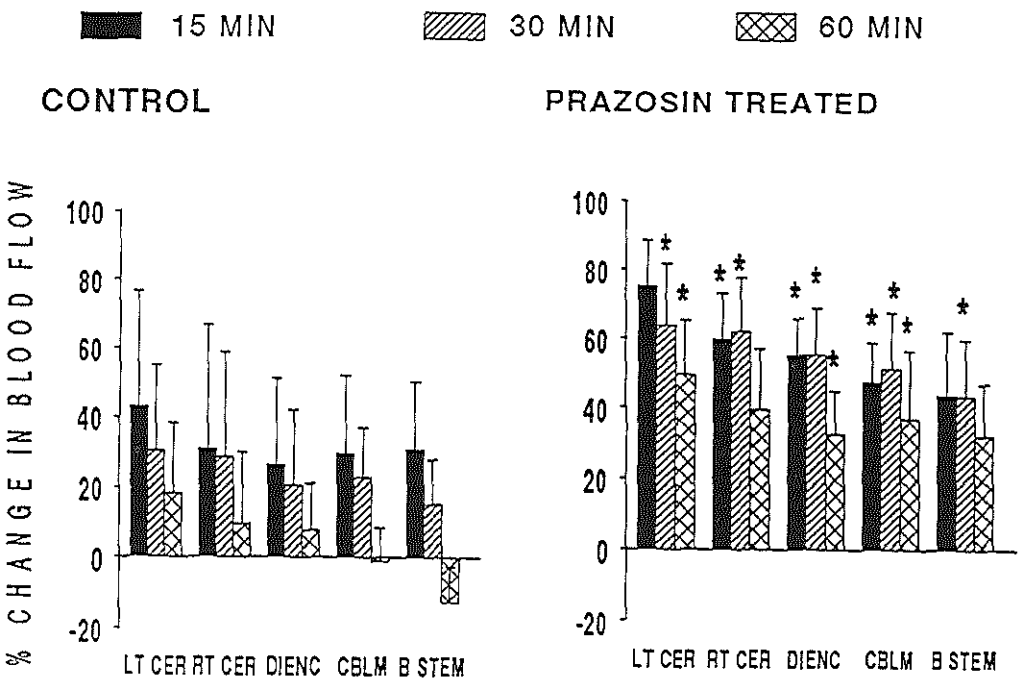

Fig. 7 The effect of DCLHb (400 mg/kg, i.v.; N= 8) on the percentage change in the blood flow to the left cerebral hemisphere (LT CER), right cerebral hemisphere (RT CER), diencephalon (DIENC), cerebellum (CBLM) and brain stem (B STEM) of control (untreated) and prazosin (1 mg/kg, i.v.; N= 5) treated rats. The measurements were made before (baseline) and 15 min, 30 min and 60 min after the administration of DCLHb to rats. Prazosin was administered 15 min prior to the administration of DCLHb. *Indicates significant difference as compared to baseline (control) or prazosin basal values (15 min after prazosin treatment).

6.4 Discussion

Blood replacement with stroma-free haemoglobin produces marked hemodynamic changes which differ from those observed with other fluids (Sharma and Gulati, 1994; Vogel *et al.*, 1986). These hemodynamic effects have been attributed to improved systemic oxygen delivery (Hauser *et al.*, 1982). However, the vascular response to stroma-free haemoglobin is believed to be independent of oxygen affinity (Nees *et al.*, 1978). Stroma-free haemoglobin solutions have been reported to produce a pressor effect through unknown mechanisms (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989; Kida *et al.*, 1991). DCLHb has been shown to produce an increase in blood pressure in conscious and anesthetized rats (Hamilton *et al.*, 1992; Gulati and Rebello, 1994). Phosphoramidon, an inhibitor of pro-endothelin conversion to endothelin, attenuated the elevation of blood pressure by DCLHb in rats, indicating that the pressor effect of DCLHb might be mediated through endothelin mechanisms (Schultz *et al.*, 1993). L-arginine, the substrate for NO, and nitroglycerine, a NO donor, significantly reduced the pressor effect of DCLHb when infused in rats 15 min after the administration of DCLHb (Schultz *et al.*, 1993). DCLHb was found to produce an increase in blood pressure in intact and cervical sectioned or bilateral adrenal demedullated rats (Gulati and Rebello, 1994). DCLHb administration in rats produced an increase in the pressor responses to norepinephrine, phenylephrine and clonidine, indicating that DCLHb produces an increase in the pressor effect of adrenergic agonists (Gulati and Rebello, 1994). DCLHb has also been found to produce marked regional circulatory changesm (Sharma *et al.*, 1994). The blood flow to the heart, spleen, gastrointestinal tract and skin was found to be significantly increased, while blood flow to other regions was not affected (Sharma and Gulati, 1994).

DCLHb produced a significant increase in blood pressure and total peripheral resistance of control rats. DCLHb also produced an increase in blood flow to the heart, portal system, gastrointestinal tract and skin of control rats. These results are consistent with some of the studies conducted earlier. Ultrapurified, polymerized bovine haemoglobin when administered to rats produced a marked increase in blood pressure and systemic vascular resistance, while the stroke volume remained unchanged (Waschke *et al.*, 1993). Stroma-free, polymerized and pyridoxylated haemoglobin administered to calves produced an increase in blood pressure (Schistek *et al.*, 1992), but the blood flow was not measured in this study. Unmodified human stroma-free haemoglobin solution when administered to dogs produced a pressor effect, but the cardiac output remained unchanged. Blood flow was found to increase in the heart, brain, liver, gut and kidney 30 min after the administration of haemoglobin solution. The glomerular filtration rate decreased and the urine flow was reduced (Ning *et al.*, 1992). Since the blood flow to the kidneys is increased and there is no change in cardiac output, it is difficult to explain the factors responsible for the decrease in the glomerular filtration rate. In the present study the

cardiac output was not significantly altered by DCLHb and blood flow was increased in the heart and GIT, but not in the liver, brain and kidneys. The reasons for these differences could be due to the differences in the species, the type of haemoglobin, the molecular modification of haemoglobin, the administration of haemoglobin and/or the purity of haemoglobin solutions.

Since adrenergic mechanisms are involved in the pressor effect of DCLHb (Gulati and Rebello, 1994) and in the regulation of blood pressure (Hoffman and Lefkowitz, 1990). DCLHb was administered in prazosin pretreated rats to assess the effect of α_1-adrenoceptor antagonist (Weiner, 1980). The pressor effect of DCLHb was significantly attenuated, the cardiac output and stroke volume were significantly increased and total peripheral resistance was significantly decreased in prazosin treated rats receiving DCLHb as compared to rats infused with DCLHb alone. However, DCLHb produced a significant increase in the blood flow to the heart, gastrointestinal tract, skin, brain, portal system, kidneys and musculo-skeletal system of prazosin treated rats. Thus, the pressor effect of DCLHb was attenuated but the increase in blood flow induced by DCLHb was maintained after prazosin pretreatment. The results of the present study are supported by earlier observations showing evidence that the pressor effect of DCLHb can be reversed by prazosin (Malcolm *et al.*, 1992). However, this is the first study demonstrating that the increase in blood flow induced by DCLHb can be maintained while the pressor effect can be attenuated by prazosin pretreatment.

Postsynaptic vascular α-adrenoceptors are present in several blood vessels and play an important role in the regulation of regional blood flow. In the heart, α_1-adrenoceptors have been demonstrated on the large epicardial coronary arteries (Heusch *et al.*, 1984). Efferent renal nerve stimulation in the dog produces vasoconstriction via activation of α_1-adrenoceptors (Osborn, 1983). The mesenteric arterial bed has been shown to possess α_1-adrenoceptors, since vasoconstrictor responses to norepinephrine could be blocked by prazosin but not by yohimbine (Nicholes and Hiley, 1985). If α_1-adrenoceptors are responsible for vasoconstriction in most of the vascular beds, DCLHb should produce a decrease in blood flow to most of the organs due to its capability to potentiate the pressor responses of α_1-adrenoceptor agonists. However, the present study shows that DCLHb increases the blood flow to most of the organs, and that the increase in blood flow could not be blocked by an α_1-adrenoceptor antagonist, prazosin. It could be possible that the major mechanism responsible for the blood flow effects of DCLHb is not via α_1-adrenoceptors but through other mechanisms like endothelin or EDRF/NO, while the major mechanism responsible for the pressor effect is through α_1-adrenoceptors. Thus, the pressor effect of DCLHb could be blocked by prazosin pretreatment but regional circulatory effects were not affected by α_1-adrenoceptor antagonist, prazosin.

It can be concluded that: (1) The pressor effect of DCLHb is associated with an increase in blood flow to various organs. (2) The pressor effect of DCLHb can be attenuated by prazosin

pretreatment. (3) The increase in blood flow induced by DCLHb is maintained in prazosin pretreated rats.

6.5 Acknowledgments

The authors would like to thank (1) Dr. P.R. Saxena from Erasmus University, Rotterdam, The Netherlands for providing the software for the calculations involved in radioactive microsphere technique, (2) Baxter Healthcare Corp. for providing financial assistance.

Chapter 7

Yohimbine modulates diaspirin crosslinked haemoglobin induced systemic hemodynamics and regional circulatory effects

Summary

Diaspirin crosslinked haemoglobin (DCLHb™; Baxter Healthcare Corp., Round Lake, IL, USA), a haemoglobin-based blood substitute is proposed to be an effective resuscitative solution. It produces an immediate, but limited increase in blood pressure when administered to conscious or anesthetized rats. This vasoactivity is associated with an increase in blood flow to several major organs. It has been shown that α-adrenoceptors in the peripheral vascular system are sensitized by DCLHb in rats. The present study was conducted to determine the effect of yohimbine, an α_2-adrenoceptor antagonist on systemic hemodynamics and regional circulatory effects of DCLHb. The systemic hemodynamics and regional circulation were measured using a radioactive microsphere technique. DCLHb (400 mg/kg, iv) produced an increase in blood pressure and total peripheral resistance, while heart rate, cardiac output and stroke volume were not significantly altered in control rats. In yohimbine (2 mg/kg, iv) pretreated animals, DCLHb did not produce any change in heart rate, stroke volume, cardiac output and total peripheral resistance, but a slight increase in blood pressure was observed compared to baseline values obtained after the administration of yohimbine. The increase in blood pressure induced by DCLHb was significantly blocked by pretreatment with yohimbine. Yohimbine (2 mg/kg, iv) per se decreased blood pressure, while other systemic hemodynamics parameters were not affected. DCLHb increased blood flow to the heart, gastrointestinal tract (stomach, small intestine, caecum and large intestine), portal (spleen, mesentery and pancreas) and skin, while blood flow to the brain (cerebral hemispheres, diencephalon, cerebellum and brain stem), liver, kidneys and musculo-skeletal system was not affected in control rats. In yohimbine pretreated animals, DCLHb produced an increase in blood flow to the heart, brain (cerebellum and brain stem), liver, small intestine, caecum, spleen, mesentery and pancreas, kidneys, skin and musculo-skeletal system, while blood flow to the stomach and large intestine was not affected. Yohimbine pretreatment significantly attenuated the DCLHb induced increase in blood flow to the large intestine, mesentery and pancreas. It is concluded that the cardiovascular actions of DCLHb are partially mediated through α_2-adrenoceptors. Adrenergic antagonists may be useful in attenuating the pressor effect of DCLHb while maintaining the regional perfusion.

7.1 Introduction

DCLHb is a blood substitute derived from the haemoglobin of outdated erythrocytes which is produced by crosslinking molecular haemoglobin between the α-subunits by a reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). DCLHb is

a highly purified haemoglobin based blood substitute (Estep *et al.*, 1989b; Estep *et al.*, 1989a) and has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). The purification process includes virus inactivation by heat pasteurization (Estep *et al.*, 1989b; Estep *et al.*, 1989a). The crosslinking of the α subunits affords DCLHb a favorable oxygen dissociation curve (Snyder *et al.*, 1987; Vandegriff *et al.*, 1989).

It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions are not adversely affected (Hess *et al.*, 1989), in contrast with results obtained previously with stroma free haemoglobin preparations. DCLHb was also found to be a promising resuscitative fluid after hemorrhage (Przybelski *et al.*, 1990). Resuscitation with DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters in rats subjected to a 20 ml/kg hemorrhage (Przybelski *et al.*, 1991). DCLHb has been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion in rats (Cole *et al.*, 1992). These studies clearly indicated that DCLHb may be of immense therapeutic value in clinical situations such as hemorrhagic shock and cerebral ischemia.

The hemodynamic response to blood replacement with unmodified stroma-free haemoglobin differs from that observed with other fluids (Vogel *et al.*, 1986), differences which have been attributed to improved systemic oxygen delivery (Hauser *et al.*, 1982). Systemic hemodynamic alterations have been reported with haemoglobin solutions including a rapid and sustained increase in mean arterial pressure (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). Several studies (Rabinovici *et al.*, 1989; Hamilton *et al.*, 1992) have recently been published demonstrating the cardiovascular actions associated with DCLHb. DCLHb (125-4000 mg/kg), when administered intravenously, produced a 25-35 % increase in mean arterial pressure in unanesthetized rats. The pressor effect of DCLHb was self limiting and was not found to be due to volume load or oncotic pressure (Hamilton *et al.*, 1992). DCLHb has also been shown to produce an increase in blood pressure of urethane anesthetized rats (Gulati and Rebello, 1994; Sharma and Gulati, 1994). It has been observed that the pressor effect of DCLHb is not mediated through the central nervous system, but appears to be mediated through the peripheral vascular system (Gulati and Rebello, 1994). It was also found that DCLHb potentiates the pressor responses of norepinephrine, phenylephrine and clonidine, indicating an increase in the sensitivity of peripheral vascular α-adrenoceptors (Gulati and Rebello, 1994). The pressor effect of DCLHb could be reversed by administering prazosin, an α_1-adrenoceptor antagonist (Malcolm *et al.*, 1992). It appears that the pressor effect of DCLHb is mediated at least in part through α-adrenoceptors. Besides producing an increase in blood pressure, DCLHb (400 mg/kg, i.v.) also produced significant alterations in the regional blood circulation of rats (Sharma and Gulati,

1994). It is well known that α-adrenoceptors are present throughout the vascular system. It could therefore be possible that the systemic hemodynamics and regional circulatory effects of DCLHb are mediated through α-adrenoceptors. The present study was carried out to determine the effect of an α_2-adrenoceptor antagonist, yohimbine, on DCLHb-induced regional circulatory and systemic hemodynamic effects.

7.2 Materials and Methods

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI, USA) weighing 300-350 g were used in the study according to the protocol approved by the University Animal Care Committee. Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a P23 ID (Gould Inc., Tst & Mgmt. Rec. Syst. Div., Valley View, OH, USA) pressure transducer for recording the blood pressure on a P7D polygraph (Grass Instrument Co., Quincy, MA, USA) through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant, and to avoid the effect of respiration on cardiovascular parameters, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a rodent ventilator (Model 683; Harvard Apparatus, Inc. S. Natick, MA, USA). The carotid artery of the right side was exposed and a PE 50 cannula was guided through the common carotid artery into the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P 23 DC pressure transducer. When the cannula reached the ventricle, the diastolic pressure dropped to zero. The femoral artery of the right side was cannulated and connected to a withdrawal pump (Model 22; Harvard Apparatus, Inc. S. Natick, MA, USA).

Determination of systemic hemodynamics and regional circulation

At each measurement, a thoroughly mixed suspension of approximately 200,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA, USA) in 0.2 ml saline was injected into the left ventricle and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of each experiment, animals were sacrificed with an overdose of pentobarbital sodium and all the tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: heart, liver, stomach, small intestine, caecum, large intestine, mesentery and pancreas, spleen, left kidney, right kidney, cerebral

hemispheres, midbrain, cerebellum, brain stem, skin, and rest of the body consisting of muscles and bones. The radioactivity in the standards, the blood samples and the tissue samples was counted in a gamma counter (Minaxi Auto-Gamma 5000 series; Packard Instrument Co. Downers Grove, IL, USA) with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output, (2) stroke volume, (3) total peripheral resistance, (4) regional blood flow [(withdrawal rate of arterial blood sample x radioactivity in tissues)/radioactivity in sampled arterial blood] and (5) regional vascular resistance (mean arterial pressure/regional blood flow). The results were calculated using the programs described earlier (Saxena *et al.*, 1980).

Administration of saline or Ringer lactate (4 ml/kg, i.v.; N=8) did not affect the systemic hemodynamics and regional blood circulation 15, 30 and 60 min after infusion. The effect of DCLHb (400 mg/kg, i.v.) in control (untreated) and yohimbine (2 mg/kg) treated rats on systemic hemodynamics and regional circulation was studied. In treated rats, DCLHb was administered 15 min after the administration of yohimbine. The dose of DCLHb was selected on the basis of studies conducted previously (Hamilton *et al.*, 1992; Gulati and Rebello, 1994; Sharma and Gulati, 1994), which demonstrated that the infusion of this dose of DCLHb resulted in a near maximal response.

Drugs

Yohimbine was purchased from Sigma Chemical Co., St. Louis, MO, USA. Diaspirin crosslinked haemoglobin in lactated electrolyte was provided by Baxter Healthcare Corporation, Round Lake, IL, USA. Yohimbine was dissolved in ethanol and diluted with normal saline and was prepared fresh at the time of each experiment.

Statistics

All data are presented as the mean value ± 1 SEM. Mean arterial blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Duncan's test or by paired t test (two tailed). Each group consisted of 6 to 8 animals and the level of $P < 0.05$ was considered significant.

7.3 Results

Effect of yohimbine pretreatment on DCLHb induced changes in systemic hemodynamics

DCLHb (400 mg/kg, i.v.) produced a significant increase in mean arterial blood pressure ($F(3,28) = 10.78$; $P < 0.0001$) at 15, 30 and 60 min and total peripheral resistance ($F(3,28) = 6.41$; $P < 0.001$) at 30 and 60 min after its administration to control (untreated) rats (table 1). Heart rate, cardiac output and stroke volume remained unaltered after the administration of DCLHb to control rats. Yohimbine (2 mg/kg, iv) decreased the mean blood pressure, while heart

rate, stroke volume, cardiac output and total peripheral resistance were not affected. When administered to yohimbine pretreated rats, DCLHb produced an increase in mean blood pressure ($F(4,25) = 8.74$, $P = 0.001$) compared to the baseline obtained following yohimbine treatment. However, the pressor effect of DCLHb was found to be significantly ($F(7,62) = 8.74$; $P < 0.0001$) less in yohimbine treated rats compared to control rats. Heart rate, cardiac output, stroke volume and total peripheral resistance were not significantly altered by DCLHb in yohimbine pretreated rats as compared to the baseline obtained following yohimbine treatment (table 1).

Effect of yohimbine pretreatment on DCLHb induced changes in brain circulation

DCLHb did not affect blood flow to the cerebral hemispheres, diencephalon, cerebellum or brain stem, while vascular resistance was significantly increased in the cerebral hemispheres ($P < 0.01$), diencephalon ($P < 0.005$), cerebellum ($P < 0.001$) and brain stem ($P < 0.005$) (fig. 1). However, in yohimbine treated rats blood flow to the cerebral hemispheres, diencephalon, cerebellum and brain stem was significantly ($P < 0.01$) increased, while vascular resistance was not affected by DCLHb (fig. 1). The increase in blood flow induced by DCLHb to the cerebellum ($F(1,12) = 10.96$; $P = 0.006$) and brain stem ($F(1,12) = 12.54$; $P = 0.004$) of yohimbine treated rats was found to be significantly greater than the blood flow to those tissues in control rats. Similarly, the increase in vascular resistance induced by DCLHb was found to be significantly blocked by yohimbine pretreatment in the cerebral hemispheres ($F(1,12) = 8.19$; $P = 0.01$), diencephalon ($F(1,12) = 11.27$; $P = 0.005$), cerebellum ($F(1,12) = 27.70$; $P = 0.0002$) and brain stem ($F(1,12) = 21.21$; $P = 0.0006$) (fig. 1). Basal blood flow was 84 ± 14 ml/min/100g in cerebral hemispheres, 92 ± 10 ml/min/100g in diencephalon, 85 ± 7 ml/min/100g in cerebellum, and 96 ± 9 ml/min/100g in brain stem.

Effect of yohimbine pretreatment on DCLHb induced changes in coronary circulation

DCLHb significantly increased blood flow to the heart ($P < 0.0001$) at 15 (+83 %), 30 (+73 %) and 60 (+48 %) min after its administration to control rats, while no significant changes were observed in vascular resistance (fig. 2). When administered to yohimbine treated rats, DCLHb increased blood flow to the heart ($P = 0.02$) at 15 (+56 %), 30 (+67 %) and 60 (+78 %) min after its administration. Thus, the increase in blood flow to the heart induced by DCLHb was not affected by yohimbine pretreatment. The vascular resistance in the heart was not significantly affected by DCLHb administration to yohimbine treated rats when compared with the baseline obtained following yohimbine treatment (fig. 2). Basal blood flow was 299 ± 47 ml/min/100g in the heart.

Effect of yohimbine pretreatment on DCLHb induced changes in renal circulation

Infusion of DCLHb did not affect blood flow to the kidneys, while vascular resistance was found to be significantly ($F(5,36) = 5.29$; $P < 0.001$) increased at 15, 30 and 60 min after its administration to control rats (fig. 3). When administered to yohimbine pretreated rats, DCLHb induced no change in vascular resistance 15 and 30 min after infusion but an increase was

observed at 60 min. Blood flow to the kidneys was significantly (P = 0.001) decreased by yohimbine treatment. Infusion of DCLHb produced a significant (P < 0.05) increase in blood flow to the kidneys when compared with the baseline obtained following yohimbine treatment (fig. 3). Basal blood flow was 327 ± 34 ml/min/100g in the left kidney and 338 ± 38 ml/min/100g in the right kidney.

TABLE 1 Effect of intravenous infusion of DCLHb (400 mg/kg) on systemic hemodynamic parameters of control and yohimbine (2 mg/kg, i.v.) pretreated rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)				
Control	357 ± 7	374 ± 7	383 ± 7	393 ± 7
Yohimbine	403 ± 10	403 ± 10	407 ± 12	420 ± 7
Blood pressure (mmHg)				
Control	78 ± 4	119 ± 8*	127 ± 7*	118 ± 8*
Yohimbine	69 ± 3	92 ± 5*#	94 ± 4*#	93 ± 3*#
Cardiac output (ml/min)				
Control	118 ± 6	139 ± 10	127 ± 5	108 ± 8
Yohimbine	91 ± 10	128 ± 15	109 ± 11	106 ± 15
Stroke volume (ml)				
Control	0.34 ± 0.01	0.37 ± 0.03	0.33 ± 0.02	0.28 ± 0.02
Yohimbine	0.22 ± 0.02	0.32 ± 0.04	0.27 ± 0.03	0.25 ± 0.04
Total peripheral resistance (mmHg/l/min)				
Control	675 ± 49	882 ± 77	1018 ± 68*	1119 ± 101*
Yohimbine	807 ± 94	778 ± 103	911 ± 114	976 ± 143

*Indicates significant (P < 0.05) difference as compared to baseline and #indicates significant (P < 0.05) difference as compared to control group.

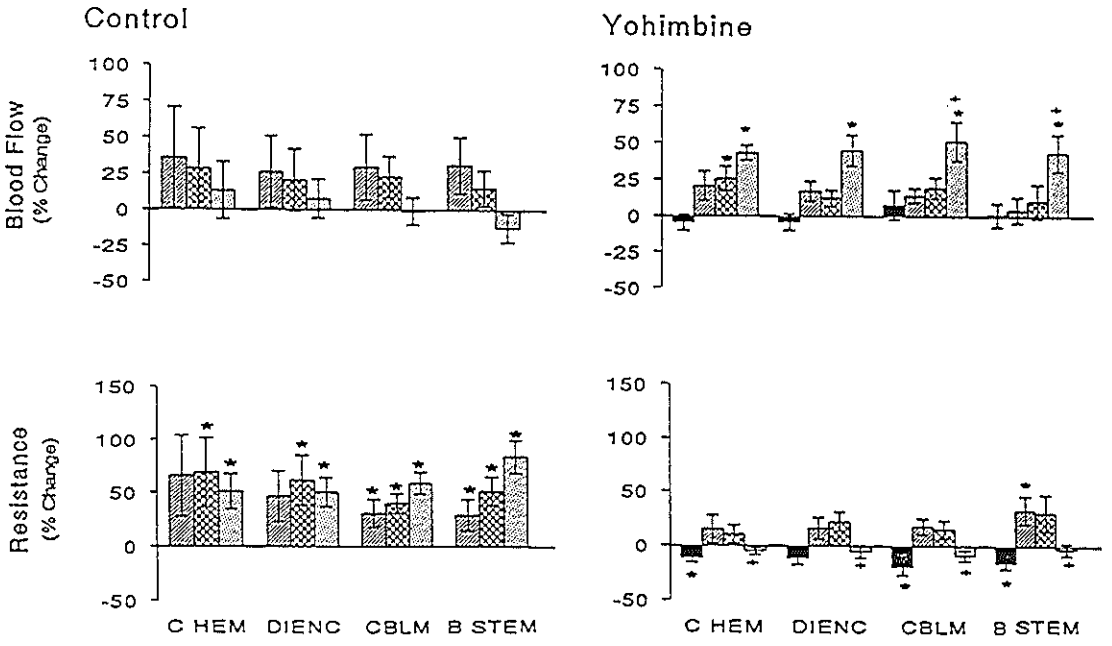

Fig. 1 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow (percent change) and the vascular resistance (percent change) in various regions of the brain (C HEM = cerebral hemispheres; DIENC = diencephalon; CBLM = cerebellum; B STEM = brain stem) of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; solid bars) treated rats. The measurements were made before (baseline), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline and +indicates significant difference compared to control.

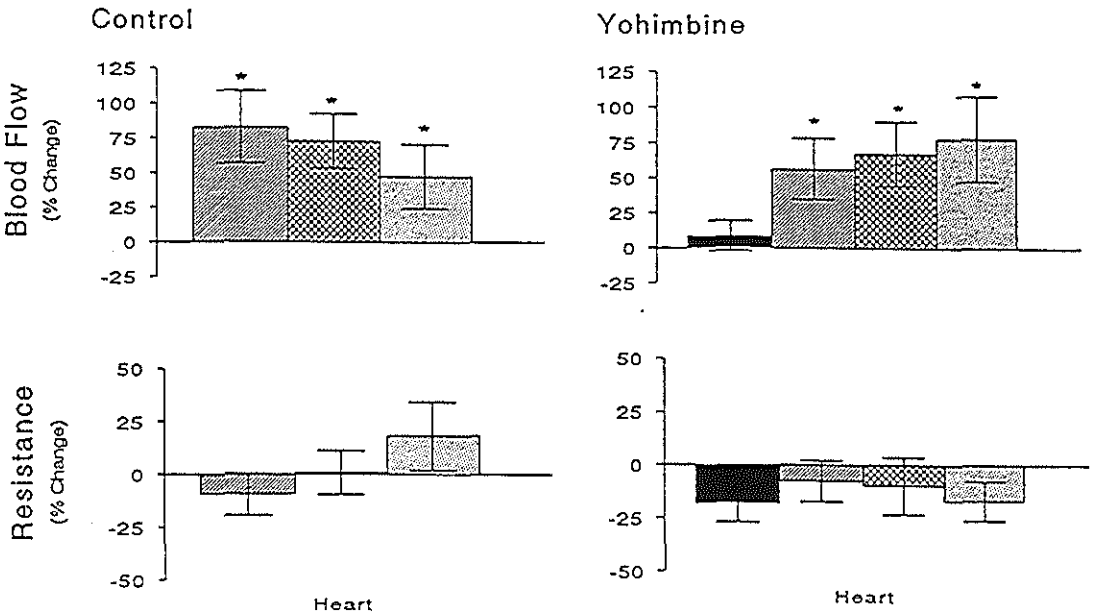

Fig. 2 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow (percent change) and the vascular resistance (percent change) in the heart of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; solid bars) treated rats. The measurements were made before (baseline), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline.

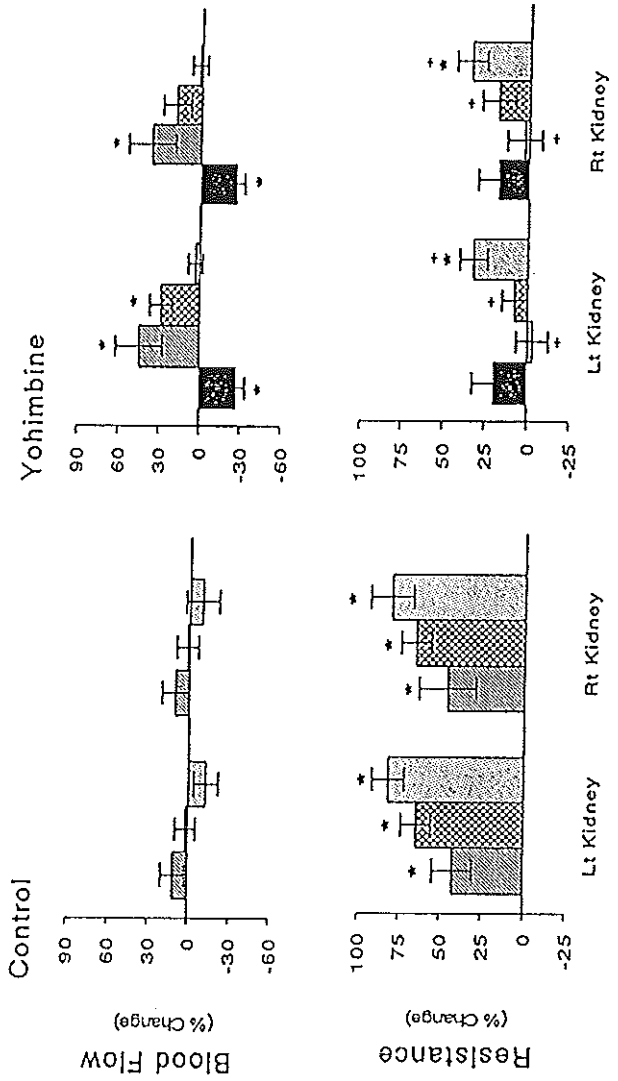

Fig. 3 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow (percent change) and the vascular resistance (percent change) in the kidneys of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; solid bars) treated rats. The measurements were made before (baseline), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline and +indicates significant difference compared to control.

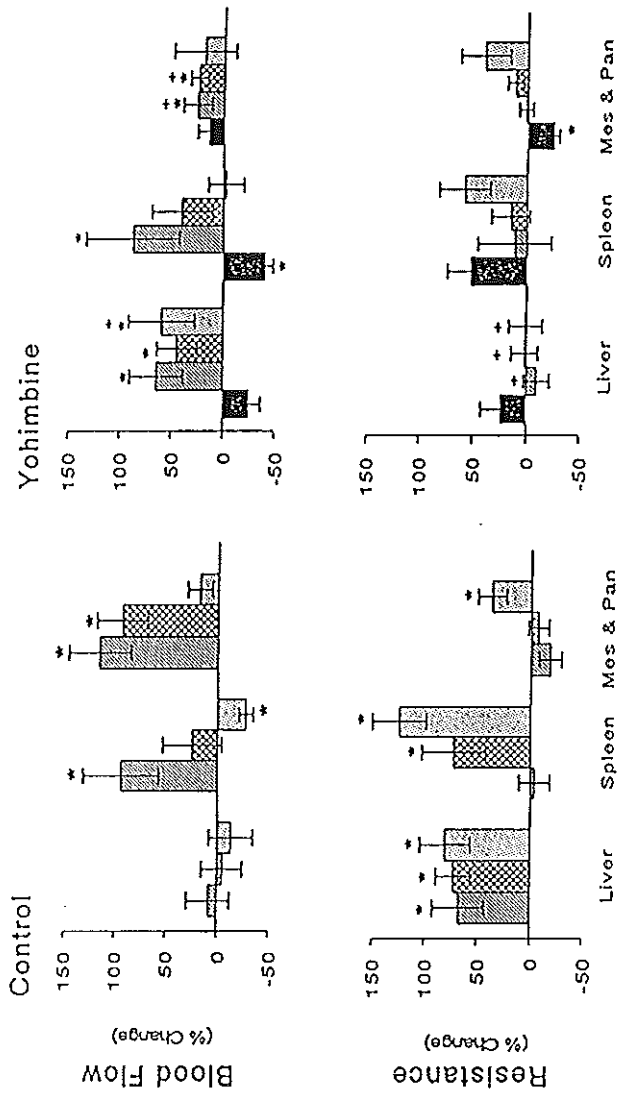

Fig. 4 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow (percent change) and the vascular resistance (percent change) in the portal system of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; solid bars) treated rats. The measurements were made before (baseline), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline and *indicates significant difference compared to control.

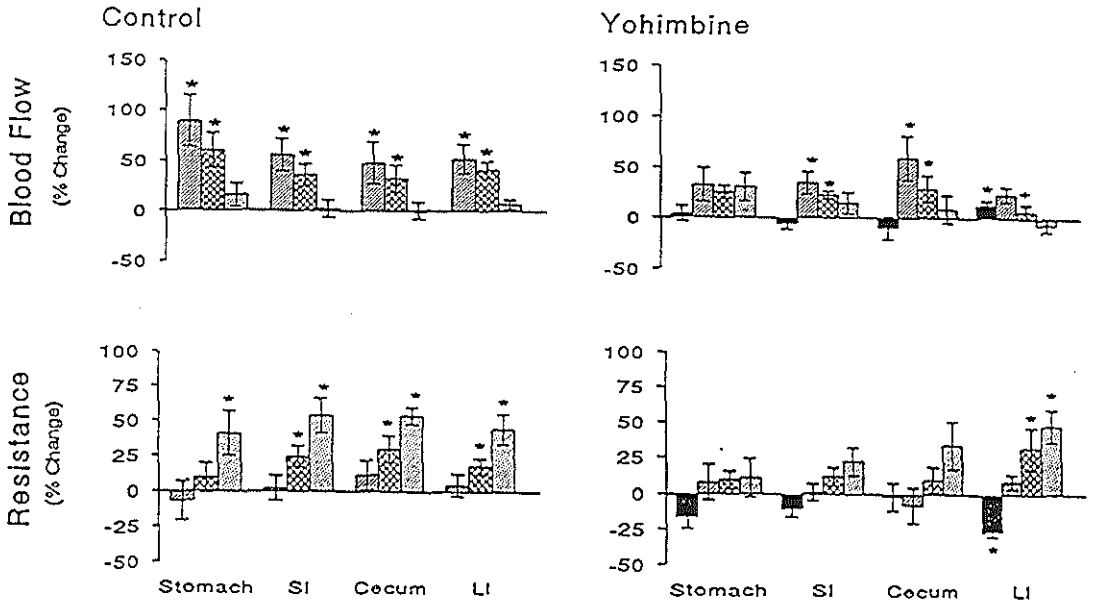

Fig. 5 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow (percent change) and the vascular resistance (percent change) in the gastrointestinal tract (stomach, small intestine (SI), caecum and large intestine (LI)) of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; solid bars) treated rats. The measurements were made before (baseline), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline and 'indicates significant difference compared to control.

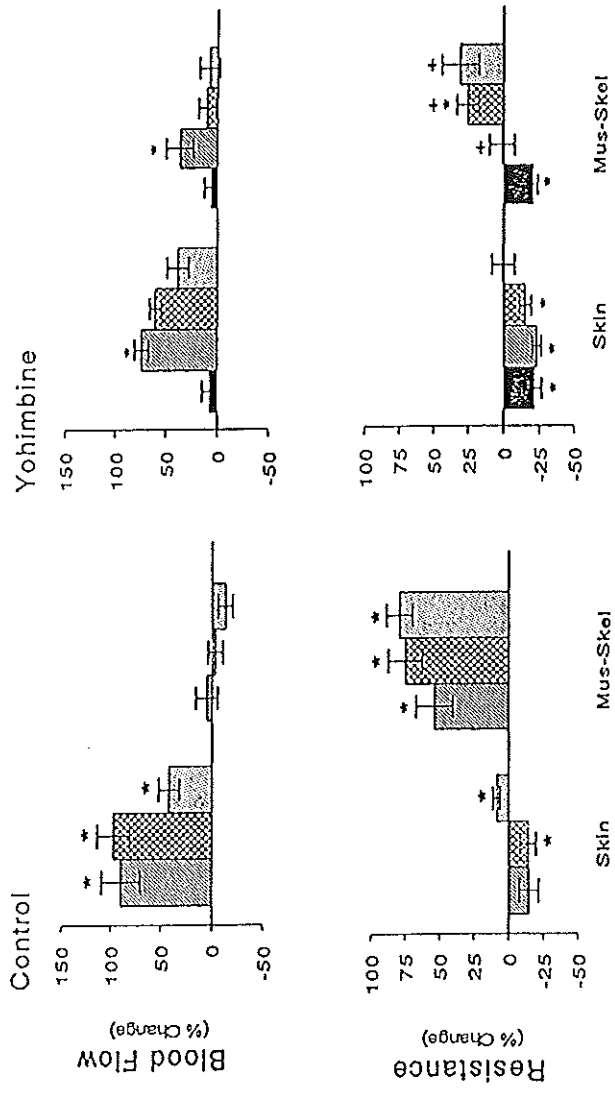

Fig. 6 The effect of DCLHb (400 mg/kg, i.v.) on the blood flow (percent change) and the vascular resistance (percent change) in the skin and musculo-skeletal system of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; solid bars) treated rats. The measurements were made before (baseline), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline and +indicates significant difference compared to control.

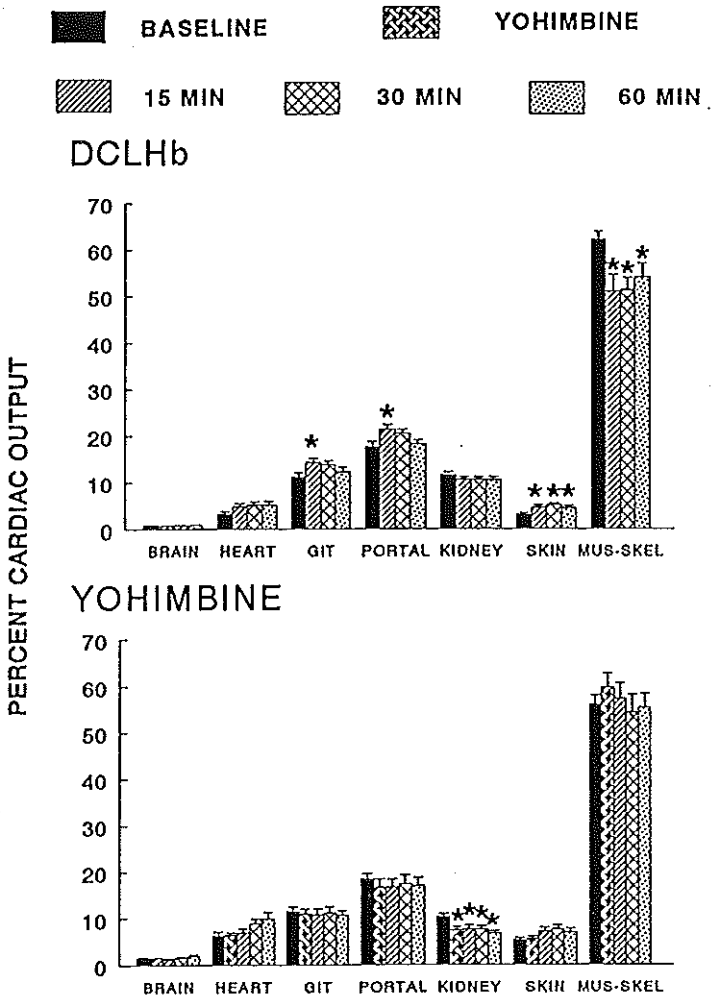

Fig. 7 The effect of DCLHb (400 mg/kg, i.v.) on the distribution of cardiac output (percent change) to various organs of control (N=8) and yohimbine (2 mg/kg, i.v.; 15 min before the administration of DCLHb; N=6; filled cross-hatched bars) treated rats. The measurements were made before (baseline; solid bars), and 15 min (hatched bars), 30 min (cross-hatched bars) and 60 min (dotted bars) after the administration of DCLHb to rats. *Indicates significant difference compared to baseline.

Effect of yohimbine pretreatment on DCLHb induced changes in portal circulation

DCLHb significantly increased blood flow to the spleen ($P < 0.01$) 15 min after infusion, and the mesentery and pancreas ($P < 0.005$) 15 and 30 min after infusion. There was no change in blood flow to the liver after DCLHb administration to control rats. Blood flow to the spleen decreased ($P < 0.005$) 60 min after the administration of DCLHb (fig 4). Vascular resistance was found to be increased in the liver ($P < 0.005$) at 15, 30 and 60 min, spleen ($P < 0.001$) at 30 and 60 min and pancreas and mesentery ($P < 0.05$) at 60 min after administration of DCLHb to control rats. Administration of yohimbine decreased blood flow to the spleen ($P < 0.001$). Blood flow to the liver and pancreas and mesentery was not affected, while vascular resistance was significantly decreased in the pancreas and mesentery ($P < 0.005$). When administered to yohimbine treated animals, DCLHb produced an increase in blood flow to the spleen ($P < 0.05$), liver ($P < 0.05$), and mesentery and pancreas ($P < 0.05$), while no significant change in vascular resistance was observed in the spleen, liver, or mesentery and pancreas. Blood flow to the pancreas and mesentery was significantly ($F(1,12) = 5.84$; $P = 0.03$) decreased in yohimbine treated rats as compared to control rats (fig. 4). The increase in vascular resistance induced by DCLHb in the liver of control rats was significantly ($F(5,36) = 4.54$; $P = 0.002$) attenuated by yohimbine pretreatment. Basal blood flow was 27 ± 3 ml/min/100g in liver, 117 ± 10 ml/min/100g in spleen and 25 ± 5 ml/min/100g in mesentry and pancreas.

Effect of yohimbine pretreatment on DCLHb induced changes in gastrointestinal tract circulation

Infusion of DCLHb significantly increased blood flow to the stomach ($P < 0.01$), small intestine ($P = 0.005$), caecum ($P < 0.05$) and large intestine ($P = 0.01$) 15 and 30 min after administration to control rats. A significant ($P < 0.01$) increase in vascular resistance was also observed in the stomach, small intestine, caecum and large intestine at 60 min after DCLHb administration to control rats (fig 5). When administered to yohimbine treated rats, DCLHb produced an increase ($P < 0.05$) in blood flow to the small intestine, caecum and large intestine, while vascular resistance was not affected in most of the regions of gastrointestinal tract except the large intestine which showed a significant increase. The DCLHb induced increase in blood flow to the large intestine was significantly ($F(5,36) = 6.23$; $P = 0.0003$) attenuated by yohimbine pretreatment (fig 5). Basal blood flow was 39 ± 4 ml/min/100g in stomach, 143 ± 12 ml/min/100g in small intestine, 153 ± 23 ml/min/100g in coecum and 57 ± 8 ml/min/100g in large intestine.

Effect of yohimbine pretreatment on DCLHb induced changes in skin and musculo-skeletal circulation

DCLHb significantly ($P < 0.001$) increased blood flow to the skin at 15, 30 and 60 min after infusion, while blood flow to the musculo-skeletal system remained unaffected after DCLHb administration to control rats. Vascular resistance was increased after DCLHb infusion

in the skin ($P < 0.05$) and musculo-skeletal system ($P < 0.0001$) of control rats (fig. 6). Yohimbine did not affect blood flow, but decreased vascular resistance to the skin and musculo-skeletal system. When administered to yohimbine treated rats, DCLHb significantly ($P < 0.05$) increased blood flow to the skin and musculo-skeletal system. This increased was associated with a decrease in vascular resistance in the skin. The increase in vascular resistance induced by DCLHb in the musculo-skeletal system of control rats was significantly ($F(5,36) = 6.84$; $P = 0.0001$) blocked by yohimbine pretreatment (fig. 6). Basal blood flow was 5.2 ± 1.0 ml/min/100g in skin and 22.2 ± 1.6 ml/min/100g in musculo-skeletal system.

Effect of yohimbine pretreatment on DCLHb induced changes in the distribution of cardiac output

DCLHb significantly ($P < 0.05$) increased the percent cardiac output to the gastrointestinal tract, skin and portal system, decreased the fraction of cardiac output to the musculoskeletal system, and had no effect on the fractional cardiac output to the brain, heart and kidneys (fig. 7). Yohimbine treatment significantly decreased the percent cardiac output to the kidneys ($F(1,11) = 13.96$; $P < 0.0001$). All other regions remained unaffected after yohimbine treatment. When administered to yohimbine treated rats, DCLHb produced a significant ($P < 0.05$) decrease in the percent cardiac output to the kidneys (fig. 7).

7.4 Discussion

The hemodynamic response to blood replacement with stroma-free haemoglobin differs from that observed with other fluids (Vogel *et al.*, 1986) and has been attributed to improved systemic oxygen delivery (Hauser *et al.*, 1982). However, the vascular response to stroma-free haemoglobin is believed to be independent of oxygen affinity (Nees *et al.*, 1978). Although most stroma-free haemoglobin solutions have been reported to produce a pressor effect (Przybelski *et al.*, 1991; Messmer *et al.*, 1977; Rabinovici *et al.*, 1989; Kida *et al.*, 1991), the mechanism of this effect is not known. DCLHb has been shown to produce an increase in blood pressure in both conscious (Hamilton *et al.*, 1992) and anesthetized rats (Gulati and Rebello, 1994). DCLHb has also been found to produce marked regional circulatory changes (Sharma and Gulati, 1994). The present study provides evidence that α-adrenoceptors are involved in the systemic hemodynamic and regional circulatory changes induced by DCLHb. Yohimbine, an α_2-adrenoceptor antagonist (Weiner, 1980), blocked the DCLHb induced increase in blood pressure and blood flow to the gastrointestinal tract. However, yohimbine pretreatment resulted in an increase in blood flow to the liver and brain following the administration of DCLHb which was not observed in control rats.

The results of the present study are supported by earlier observations showing evidence that the pressor effect of DCLHb can be reversed by prazosin, an α_1-adrenoceptor antagonist

(Malcolm *et al.*, 1992). The pressor effect of DCLHb was not affected by cervical transection, or by bilateral adrenal demedullation, indicating that the pressor effect of DCLHb is not mediated through the central nervous system or through the release of catecholamines from the adrenal medulla (Gulati and Rebello, 1994). It appears that the peripheral vascular system is directly involved in the cardiovascular actions of DCLHb. Since α-adrenoceptors have an important role in the regulation of blood pressure (Hoffman and Lefkowitz, 1990), the role of adrenergic mechanisms in the pressor effect of DCLHb was studied. DCLHb potentiated the pressor effect of norepinephrine, phenylephrine and clonidine (Gulati and Rebello, 1994), indicating that both α_1- and α_2-adrenoceptors are involved in the pressor effect of DCLHb. These studies indicate that DCLHb increases the sensitivity of peripheral vascular α-adrenoceptors.

Traditionally, α_2-adrenoceptors have been classified as those α_2-adrenoceptors situated presynaptically, in contrast with α_1-adrenoceptors, which are located postsynaptically. Peripheral postsynaptic vascular α_2-adrenoceptors are also now known to exist (Drew and Whiting, 1979; Van Zwieten and Timmermans, 1983) and produce significant alterations in systemic hemodynamics and regional circulation (Flacke *et al.*, 1990; Schmeling *et al.*, 1991; Karlson *et al.*, 1990). The distribution of α_2-adrenoceptors varies in different tissues, and the coronary and skin blood vessels are known to possess these receptors (Hoffman and Lefkowitz, 1990). Evidence exists for the mediation of cerebral vasoconstriction by postsynaptic α_2-adrenoceptors (Tsukahara *et al.*, 1986; Kanavati *et al.*, 1986). The present study provides evidence that α_2-adrenoceptors are involved in the regional circulatory actions of DCLHb.

The increase in the sensitivity of α-adrenoceptors observed after DCLHb infusion could be either due to a direct action of DCLHb on these receptors or an indirect effect of interaction with other vasoactive mechanisms. Phosphoramidon, an inhibitor of pro-endothelin conversion to endothelin, attenuated the pressor effect of DCLHb (Schultz *et al.*, 1993). There are studies which indicate that peripheral adrenergic receptors are sensitized by endothelin. The interaction of endothelin with norepinephrine was studied with regard to the effect on the perfusion pressure in rat mesenteric arteries. Endothelin-1 at subpressor doses (10^{-11} and 10^{-10} M) enhanced the pressor response to norepinephrine (Tabuchi *et al.*, 1989). The vasoconstrictor response of norepinephrine in the rat isolated perfused tail artery was significantly potentiated by subthreshold concentrations of endothelin-1 (Reid *et al.*, 1991). Endothelin-1, at a concentration with no vasoconstrictor activity, enhanced the responses to perivascular stimulation and norepinephrine in the rabbit ear artery (Wong Dusting *et al.*, 1991). The norepinephrine responses have been found to be potentiated by endothelin in human internal mammary and left anterior descending coronary arteries (Yang *et al.*, 1990). The hypotensive effect is blocked, while the pressor effect of clonidine is potentiated, by endothelin-1 treatment (Gulati and Srimal, 1993). It could therefore be possible that DCLHb increases the synthesis/release of endothelin which in turn increases the sensitivity of α-adrenoceptors. On the other hand, if DCLHb

increases the sensitivity of α-adrenoceptors which are responsible for the regional circulatory effects of DCLHb, then the blood flow to the skin, heart and brain should be reduced and not increased following DCLHb administration as observed in the present study. It is therefore clear that other vasoactive mechanisms are also involved in regional circulatory effects of DCLHb.

Cardiovascular effects as a result of stimulation of α-adrenoceptors could be modulated by endothelium derived relaxation factor (EDRF). A basal release of EDRF may nonspecifically oppose the effect of stimulation of α_2-adrenoceptors on vascular smooth muscle, probably due to functional antagonism (Martin *et al.*, 1986). EDRF inhibits the release of norepinephrine from adrenergic nerves in rabbit carotid arteries and dog mesenteric arteries (Cohen and Weisbrod, 1988; Greenberg *et al.*, 1990). It has also been demonstrated that the DCLHb-induced elevation in mean arterial blood pressure is mediated at least in part by the EDRF/nitric oxide system. L-arginine, the substrate for nitric oxide synthesis, and nitroglycerine, a nitric oxide donor, significantly reduced the pressor effect of DCLHb (Schultz *et al.*, 1993). It is well known that nitric oxide is rapidly inactivated by haemoglobin (Gibaldi, 1993; Moncada and Higgs, 1993). Haemoglobin solutions have a direct scavenging action on nitric oxide and it is possible that DCLHb indirectly influences the adrenergic systems to produce cardiovascular actions as a consequence of the interaction of haemoglobin with the nitric oxide and/or endothelin pathways.

It is concluded that (1) DCLHb produces significant systemic hemodynamic and regional circulatory changes, (2) the increase in blood flow to the gastrointestinal tract induced by DCLHb could be blocked by yohimbine and (3) yohimbine pretreatment led to an increase in blood flow to the kidneys, liver and brain after DCLHb infusion. It is possible that the cardiovascular actions of DCLHb are mediated in part through the peripheral α_2-adrenoceptors.

7.5 Acknowledgments

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Part 4

Role of nitric oxide mechanism in the cardiovascular effects of diaspirin crosslinked haemoglobin

Chapter 8

Role of nitric oxide mechanism in the cardiovascular effects of diaspirin crosslinked haemoglobin in anesthetized rats

Summary

The role of nitric oxide (NO) in the cardiovascular actions of diaspirin crosslinked haemoglobin (DCLHb) was studied in anesthetized rats. The regional circulatory and systemic hemodynamic effects of DCLHb (400 mg/kg iv) were studied using a radioactive microsphere technique in control (untreated) and L-arginine (NO precursor) pretreated rats. DCLHb produced a significant increase in blood pressure (75%), cardiac output (42%), stroke volume (36%) and total peripheral resistance (45%) without affecting heart rate, when administered to control rats. L-arginine pretreatment significantly attenuated DCLHb-induced systemic hemodynamic effects. DCLHb induced increase in blood flow to the skin and spleen was completely and to the heart was partially blocked by L-arginine pretreatment suggesting that cardiovascular actions induced by DCLHb could be antagonized by NO precursor, L-arginine. NO synthase (NOS) inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester), produced significant increases in regional vascular resistance leading to a decrease in blood flow to all the organs excepting heart, where an increase in blood flow and decrease in vascular resistance was observed. DCLHb when administered in L-NAME pretreated rats, accentuated the decrease in blood flow to the GIT, spleen, mesentery and pancreas, skin and musculoskeletal system. These studies provide evidence that NO precursor L-arginine can attenuate the effects of DCLHb, and DCLHb can potentiate the effect of NOS inhibitor, L-NAME. The role of NO in the mechanism of action of DCLHb was further studied by estimating plasma cGMP in control, DCLHb treated, L-NAME treated and L-NAME followed by DCLHb treated rats. DCLHb and L-NAME significantly decreased the concentration of circulating cGMP in the blood plasma. L-NAME pretreatment potentiated DCLHb-induced decrease in cGMP levels. Since the formation of cGMP is stimulated by NO, these studies provide additional evidence for the involvement of NO in the mechanism of action of DCLHb. It is concluded that NO plays an important role in the cardiovascular effects of DCLHb.

8.1 Introduction

Modification of haemoglobin is being extensively carried out with the aim of developing a safe and effective blood substitute. A variety of haemoglobin solutions have been developed as resuscitative solutions (Chang, 1992). Diaspirin crosslinked haemoglobin (DCLHb) is a blood substitute derived from the haemoglobin of the human erythrocytes. DCLHb has been developed by crosslinking molecular haemoglobin between the α-subunits by a reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process

includes heat pasteurization of the solution (Estep *et al.*, 1989b; Estep *et al.*, 1989a). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). The crosslinking of the α subunits affords the haemoglobin a favorable oxygen dissociation curve (Vandegriff *et al.*, 1989). DCLHb has been found to be an effective resuscitative fluid following hemorrhage (Przybelski *et al.*, 1990). It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions were not affected significantly (Hess *et al.*, 1989). DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters to hemorrhaged rats (Przybelski *et al.*, 1991). DCLHb has also been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion in rats (Cole *et al.*, 1992).

DCLHb has also been reported to produce a rapid and sustained increase in mean arterial pressure (Gulati and Rebello, 1994; Hamilton *et al.*, 1992). Purified stroma free haemoglobin (SFHb) solutions have been reported to produce vasoconstrictor activity in a variety of experimental models (Gilroy *et al.*, 1988; Vogel *et al.*, 1986). Unmodified human SFHb was found to produce an increase in blood flow to the heart, brain, liver, gut and kidneys of dog (Ning *et al.*, 1992). Yamakawa et al. reported that SFHb solution when administered to rats, produced a marked vasodilatation of coronary blood vessels (Yamakawa *et al.*, 1990). DCLHb has also been demonstrated to produce significant increase in blood flow to the heart, gastrointestinal tract, spleen and skin and that the pressor effect is not associated with a decrease in blood flow to various organs in rats (Sharma and Gulati, 1994).

Although DCLHb produces significant effect on the cardiovascular system only a few studies have been performed to determine the mechanisms involved. Involvement of adrenergic receptors (Gulati and Rebello, 1994), endothelin (ET) and nitric oxide (NO) system (Schultz *et al.*, 1993) in the pressor effect of DCLHb has been implicated. No study has been performed to determine the mechanism involved in the regional circulatory effects of DCLHb. The aim of the present study was to determine the role of NO in regional blood circulation and systemic hemodynamic effects of DCLHb. Studies were performed to determine systemic hemodynamics and regional blood circulation in rats, pretreated with (a) L-arginine, a precursor for NO and (b) L-NAME, a NO synthase inhibitor. The effect of DCLHb on blood plasma concentration of cGMP in control and L-NAME pretreated rats was also determined.

8.2 Materials and Methods

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI) weighing 340-390 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, intraperitoneal). The left

femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant, and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration with ambient air by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683. Arterial blood pO_2, pCO_2 and pH were measured using a pH/blood gas analyzer (ABL330 Radiometer, Copenhagen, Denmark). Total haemoglobin was measured using a hemoximeter (IL482 Co-oximeter system, Instrumentation Laboratory, Lexington, MA). The carotid artery of the right side was exposed and a PE 50 cannula was guided through the common carotid artery into the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P23 DC pressure transducer. When the cannula reached the ventricle, the diastolic pressure dropped to zero. The femoral artery of the right side was cannulated and connected to a withdrawal pump (Harvard Model 22).

Determination of systemic hemodynamics and regional circulation

At each measurement, a thoroughly mixed suspension of approximately 200,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA) in 0.2 ml saline were injected into the left ventricle and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all the tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: lungs, heart, liver, stomach, small intestine, caecum, large intestine, mesentery and pancreas, spleen, left kidney, right kidney, left cerebral hemisphere, right cerebral hemisphere, midbrain, cerebellum, brain stem, skin and musculoskeletal system. The radioactivity in the standards, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output [(radioactivity injected x withdrawal rate of arterial blood) / radioactivity in sampled arterial blood], (2) stroke volume [cardiac output / heart rate], (3) total peripheral resistance [mean arterial pressure / cardiac output], (4) regional blood flow [(radioactivity in tissue x withdrawal rate of arterial blood) / radioactivity in sampled arterial blood] and (5) regional vascular resistance [mean blood pressure / regional blood flow]. Infusion of an equal volume of saline did not affect the systemic hemodynamics or regional circulation. The effect of DCLHb (400 mg/kg iv) on the systemic hemodynamics and regional circulation, both in

normal and L-arginine treated rats, was studied. L-arginine (100 mg/kg/h) infusion was started 15 min prior to the administration of DCLHb and continued throughout the experiment. L-NAME (10 mg/kg iv) was administered 15 min prior to the administration of DCLHb. The dose of DCLHb was selected on the basis of studies conducted previously (Gulati and Rebello, 1994; Hamilton *et al.,* 1992; Sharma and Gulati, 1994) which demonstrated that infusion of this dose of DCLHb resulted in a near maximal pressor response.

Determination of cGMP in the blood plasma

The plasma samples were assayed for cGMP as described earlier (Harper and Brooker, 1975). The blood samples were collected from the left femoral artery of rats, treated with either vehicle, DCLHb, L-NAME or L-NAME plus DCLHb, in plastic tubes containing EDTA (1 mg/ml) and aprotinin (500 kIU/ml). The samples were centrifuged at 3,000 x g for 15 min at 4°C and plasma was separated and assayed for cGMP. Supernatant was extracted 3 times with 5 ml of water saturated ether and then samples were evaporated to dryness using nitrogen. The residue was dissolved in 50 mM sodium acetate buffer (pH 4.75).

Aliqouts (0.5 ml) were taken from each sample after ether extraction. Acetylation was done directly on each sample at room temperature by first adding 0.01 ml of triethylamine and then 0.05 ml of acetic anhydride. Acetic anhydride was added to each sample immediately after the addition of triethylamine in order to minimize the time that the sample is exposed to basic conditions. Cyclic GMP immunoassay was performed in 50 mM sodium acetate buffer (pH 4.75) using cGMP RIA kit (Advanced Magnetics Inc., Cambridge, MA). The incubation mixture for RIA consisted of 100 µl of standards or samples, 100 µl of tracer and 100 µl of anti-serum. The tubes were vortexed and incubated overnight at 4°C. Next day, magnetic goat anti-rabbit IgG (500 µl) was added and incubated for 20 min at room temperature. The tubes were then placed in a magnetic separation unit for 10 min. The supernatant was decanted and radioactivity was determined using a Gamma Counter (Packard Instruments, Downers Grove, IL; Model Cobra 5005) and the data was calculated. The concentration of cGMP was expressed as pmoles/ml of plasma.

Drugs

Diaspirin crosslinked haemoglobin (DCLHb) was prepared and provided by Baxter Healthcare Corp., Round Lake, IL. The physico-chemical characteristics of DCLHb were as follows: haemoglobin content 10.0 to 10.2 g/dl, methaemoglobin 1.7 to 5.4 g/dl, p50 at 37°C 32 to 33 mmHg (defined as the partial pressure of oxygen at which haemoglobin is 50 % saturated), osmolality ≈290 mOsm/kg, sodium ≈140 mEq/l, potassium ≈5 mEq/l, phospholipid contents 0.2 ppm, endotoxin <0.125 EU/ml and crosslinking between α subunits is 99.9 %. L-arginine and L-NAME were purchased from Sigma Chemicals Company, St. Louis, MO. and prepared freshly on each day of the experiment.

Statistics

All data are presented as the mean values ± 1 SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. At least 7 to 10 animals were used in each group. Data were subjected to an analysis of variance followed by a Duncan's test. A level of $P < 0.05$ was considered significant.

8.3 Results

Effect of saline on systemic hemodynamics and regional blood flow

Saline (4 ml/kg iv) was administered to anesthetized rats (N = 10). The baseline blood pressure was 83.08 ± 4.21 mmHg. The blood pressure (80.70 ± 5.01 mmHg) did not change significantly ($P > 0.05$) following administration of saline. Similarly, heart rate, cardiac output, stroke volume and total peripheral resistance were not altered following saline administration, when compared with baseline. The basal blood flow to the brain (85.7 ± 7.15 ml/min/100 g tissue), heart (481 ± 63 ml/min/100 g tissue), liver (25.07 ± 2.56 ml/min/100 g tissue), spleen (83.54 ± 18.41 ml/min/100 g tissue), mesentery and pancreas (21.51 ± 5.02 ml/min/100 g tissue), gastrointestinal tract (GIT) (116.83 ± 22 ml/min/100 g tissue), kidneys (276.08 ± 27.06 ml/min/100 g tissue), skin (5.22 ± 0.91 ml/min/100 g tissue) and musculoskeletal system (16.70 ± 1.56 ml/min/100 g tissue) was found to be unaltered following saline administration. Saline administration did not produce any significant change in regional blood flow to the brain ($P > 0.05$), heart ($P > 0.05$), GIT ($P > 0.05$), liver ($P > 0.05$), spleen ($P = 0.05$), mesentery and pancreas ($P > 0.05$), skin ($P > 0.05$) and musculoskeletal system ($P > 0.05$), when compared to baseline.

Effect of DCLHb on arterial blood gases and total haemoglobin

The baseline values of pH, pO_2 and pCO_2 were found to be 7.34 ± 0.01, 105.00 ± 3.57 mmHg and 31.78 ± 2.04 mmHg, respectively. The pH, pO_2 and pCO_2 values were found to be 7.35 ± 0.03, 103.27 ± 6.34 mmHg and 34.15 ± 2.73 mmHg, respectively at 15 min, 7.37 ± 0.03, 103.03 ± 3.41 mmHg and 30.27 ± 1.44 mmHg, respectively at 30 min and 7.36 ± 0.05, 97.23 ± 7.09 mmHg and 31.12 ± 1.93 mmHg, respectively at 60 min after the administration of DCLHb (400 mg/kg iv). The total haemoglobin was 14.80 ± 0.37 g/dl at baseline and 14.70 ± 0.31 g/dl at 15 min, 13.17 ± 0.57 g/dl at 30 min and 14.37 ± 0.56 g/dl at 60 min after the administration of DCLHb. Administration of saline or DCLHb did not produce any significant change in these values.

Effect of L-arginine pretreatment on DCLHb-induced changes in systemic hemodynamics

DCLHb (400 mg/kg iv) produced a significant ($P = 0.012$) increase in the blood pressure (75%), cardiac output (42%), stroke volume (36%) and total peripheral resistance (45%), while

heart rate was not affected when administered to control rats. However, infusion of DCLHb in L-arginine pretreated rats did not produce any significant effect on heart rate, blood pressure, cardiac output, stroke volume and total peripheral resistance (Table 1). L-arginine pretreatment significantly blocked DCLHb-induced increase in blood pressure ($P = 0.02$), cardiac output ($P = 0.04$) and stroke volume ($P = 0.05$) (Table 1). L-arginine *per se* did not produce any significant effect on the blood pressure, heart rate, stroke volume, cardiac output and total peripheral resistance, when compared to baseline values.

Effect of L-arginine pretreatment on DCLHb-induced changes in the regional blood flow and vascular resistance

DCLHb produced a significant increase in blood flow to the heart (192%), GIT (60%), spleen (105%), mesentery and pancreas (102%), and skin (73%). L-arginine, *per se*, produced an increase in blood flow to the brain, heart and GIT. In L-arginine treated rats, DCLHb produced a significant increase in blood flow to the heart (118%), GIT (68%), mesentery and pancreas (63%). However, DCLHb produced a significant increase (32%) followed by a decrease (-17%) in blood flow to the skin and a decrease in blood flow to the musculoskeletal system of L-arginine pretreated rats. L-arginine significantly attenuated DCLHb-induced increase blood flow in the heart ($P = 0.03$), spleen ($P = 0.05$), and skin ($P = 0.03$), when compared with control rats. DCLHb significantly increased regional vascular resistance in the GIT, liver, spleen, kidneys and musculoskeletal system. L-arginine *per se* decreased vascular resistance in the heart. DCLHb, in L-arginine-treated rats, produced significant decrease in vascular resistance only in the heart. DCLHb showed significant increase in vascular resistance in the liver and musculoskeletal system of L-arginine-treated rats (Figs. 1 and 2).

TABLE 1 Effect of DCLHb (400 mg/kg iv; N = 10) on the systemic hemodynamics of control and L-arginine (100 mg/kg/h iv; N = 10) treated rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)				
DCLHb	385 ± 16	401 ± 12	405 ± 12	399 ± 12
L-arginine + DCLHb	377 ± 11	376 ± 9	370 ± 12	359 ± 14
Blood pressure (mmHg)				
DCLHb	85.0 ± 4.4	129.4 ± 10.3*	119.2 ± 12.3*	108.0 ± 7.0*
L-arginine + DCLHb	80.3 ± 4.7	117.3 ± 7.4*	98.3 ± 8.2	84.7 ± 7.4^{+}
Cardiac output (ml/min)				
DCLHb	84.1 ± 5.2	113.7 ± 5.3*	110.9 ± 10.5*	82.2 ± 5.8
L-arginine + DCLHb	89.9 ± 7.8	108.1 ± 14.4	94.8 ± 13.3	64.9 ± 6.54^{+}
Stroke volume (ml)				
DCLHb	0.22 ± 0.01	0.29 ± 0.01*	0.28 ± 0.03*	0.23 ± 0.02
L-arginine + DCLHb	0.24± 0.02	0.28± 0.03	0.25 ± 0.03	0.17 ± 0.02^{+}
Total peripheral resistance (mmHg/l/min)				
DCLHb	908 ± 82	1182 ± 137	1132 ± 154	1319 ± 112*
L-arginine + DCLHb	964 ± 111	1275 ± 218	1148 ± 125	1302 ± 177

*Indicates significant ($P < 0.05$) difference compared to baseline. $^{+}$Indicates significant ($P < 0.05$) difference compared to control rats.

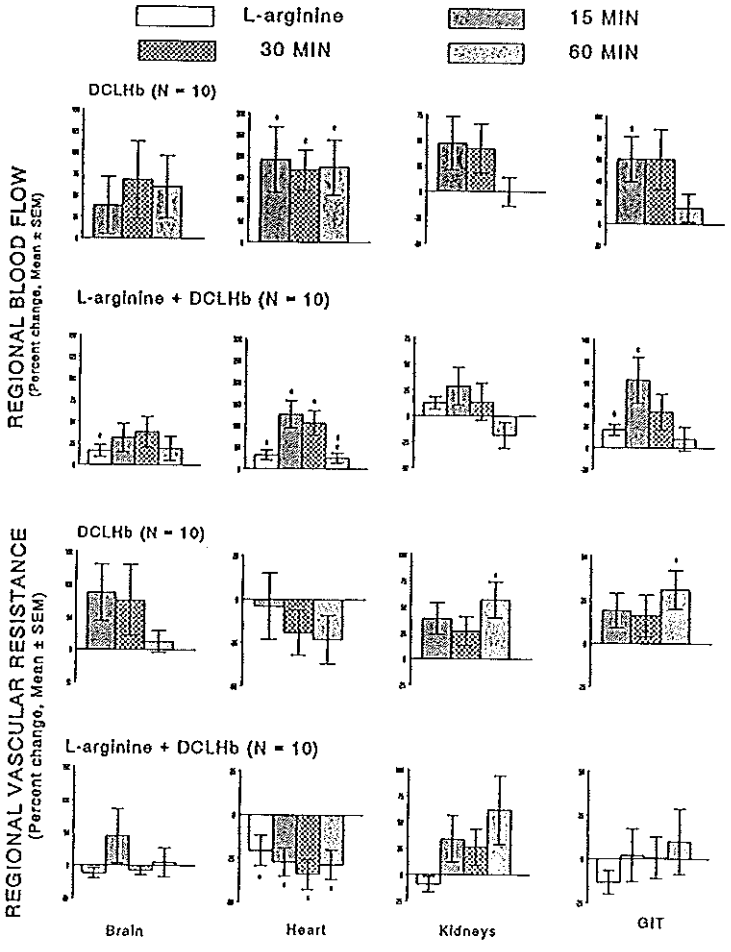

Fig. 1 The effect of DCLHb (400 mg/kg iv) on blood flow and regional vascular resistance (percent change from baseline) to the brain, heart, kidneys and gastrointestinal tract (GIT) at 15 min, 30 min and 60 min after administration to control (untreated) and L-arginine (100 mg/kg/h iv infusion starting 15 min before the administration of DCLHb) treated rats. *Indicates significant difference compared to baseline and †indicates significant difference compared to control group.

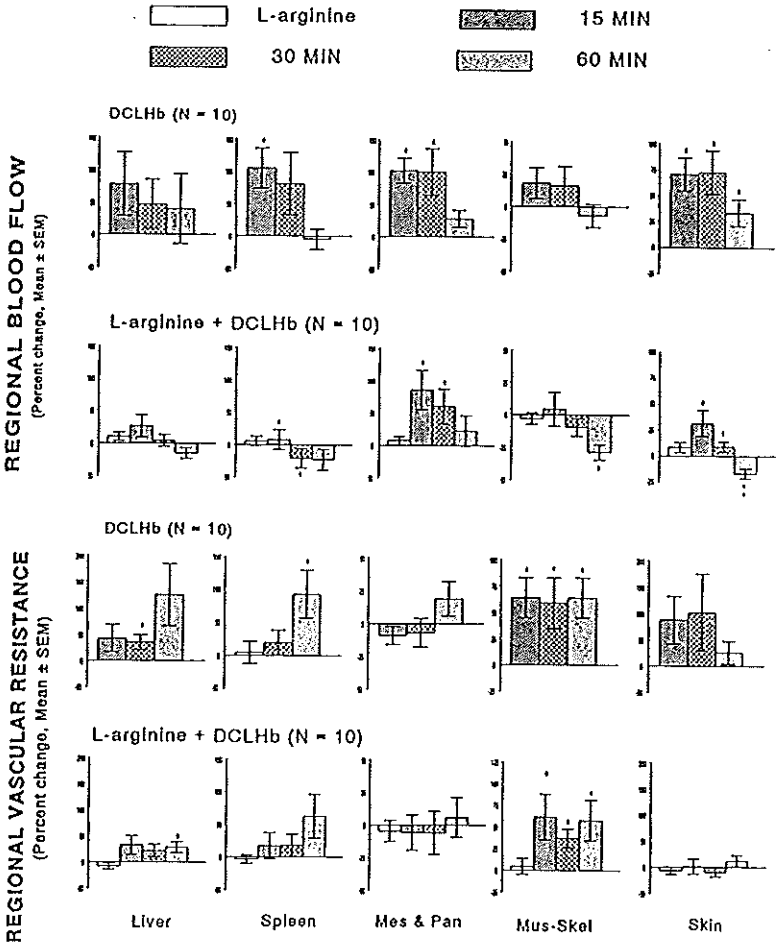

Fig. 2 The effect of DCLHb (400 mg/kg iv) on blood flow and regional vascular resistance (percent change from baseline) to the liver, spleen, mesentery & pancreas (Mes & Pan), skin and musculoskeletal (Mus-Skel) system at 15 min, 30 min and 60 min after administration to control (untreated) and L-arginine (100 mg/kg/h iv infusion starting 15 min before the administration of DCLHb) treated rats. *Indicates significant difference compared to baseline and $^{+}$indicates significant difference compared to control group.

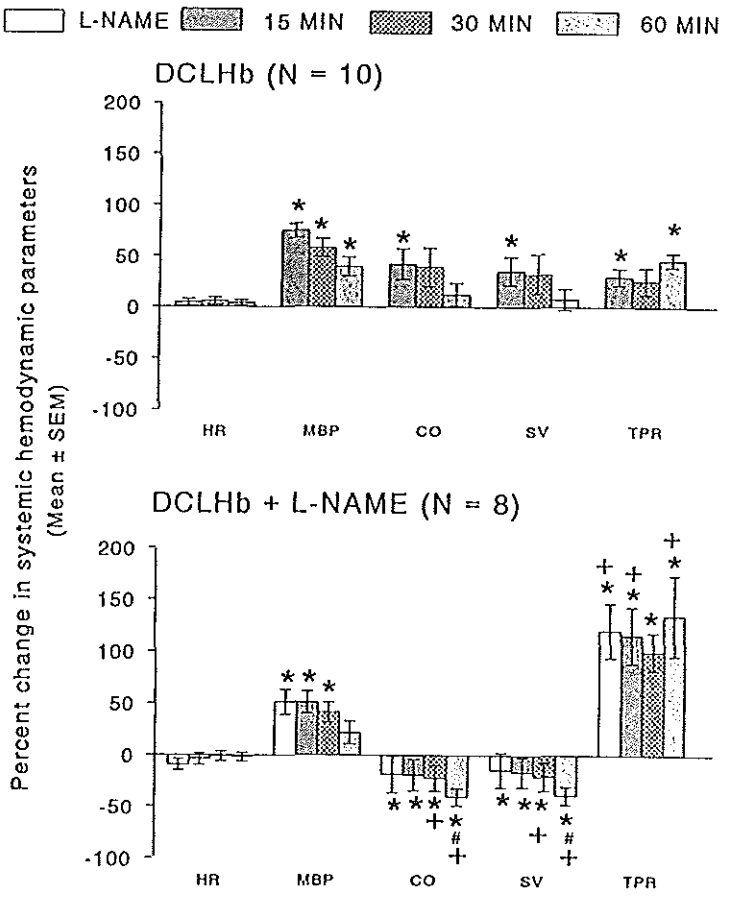

Fig. 3 The effect of DCLHb (400 mg/kg iv) on heart rate (HR), mean blood pressure (MBP), cardiac output (CO), stroke volume (SV) and total peripheral resistance (TPR) (percent change from baseline) at 15 min, 30 min and 60 min after its administration to control and L-NAME (10 mg/kg iv 15 min before the administration of DCLHb) treated rats. *Indicates significant difference compared to baseline, # indicates significant difference compared to values obtained 15 min after L-NAME and +indicates significant difference compared to control group.

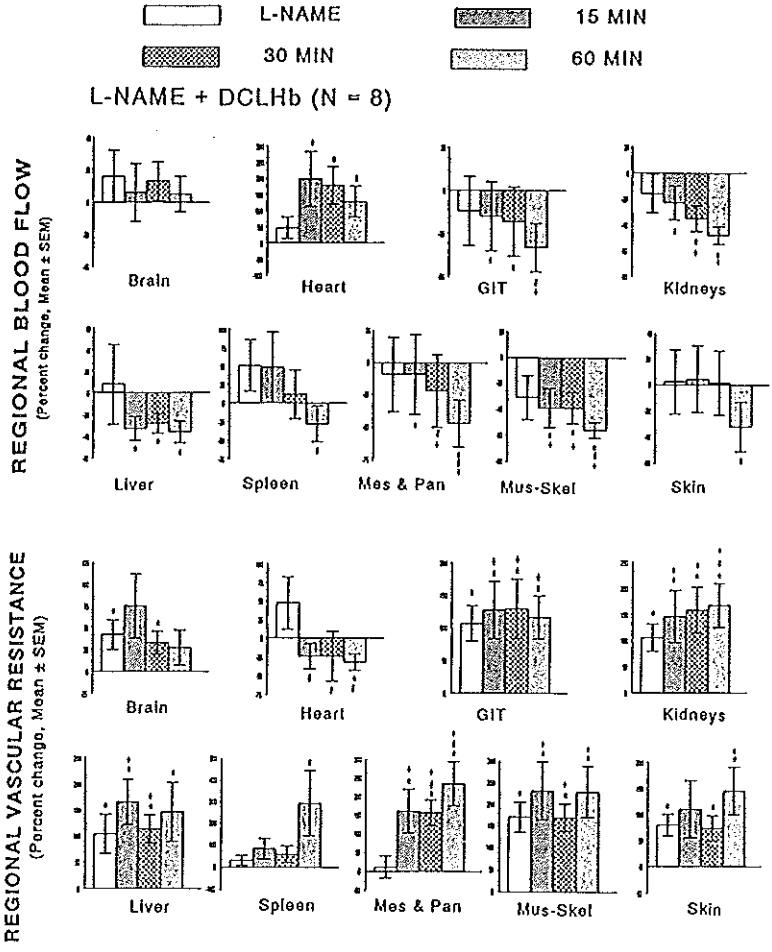

Fig. 4 The effect of DCLHb (400 mg/kg iv) on blood flow and vascular resistance (percent change from baseline) to the brain, heart, gastrointestinal tract (GIT), kidneys, liver, spleen, mesentery & pancreas (Mes & Pan), musculoskeletal (Mus-Skel) system and skin at 15 min, 30 min and 60 min after its administration to L-NAME (10 mg/kg iv 15 min before the administration of DCLHb) treated rats. *Indicates significant difference compared to baseline, # indicates significant difference compared to values obtained 15 min after L-NAME, and +indicates significant difference compared to control group.

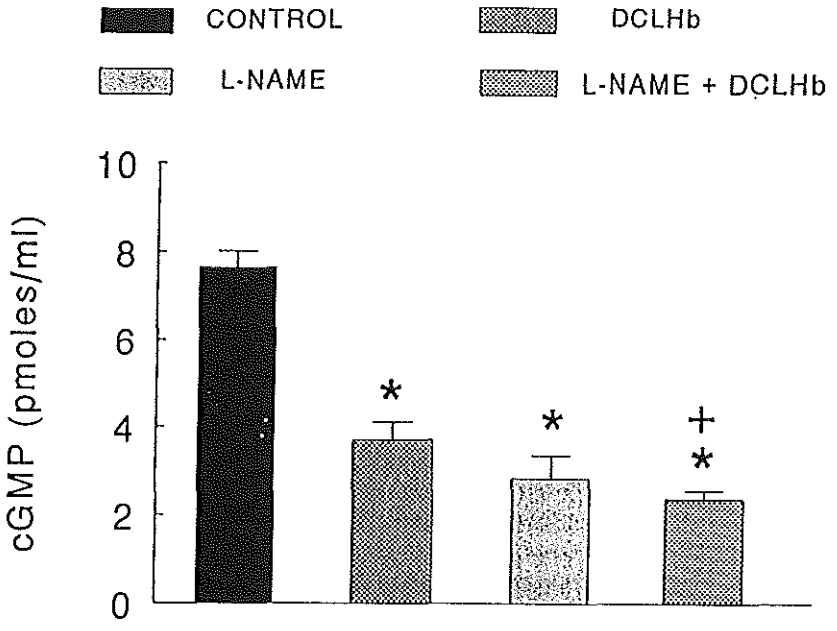

Fig. 5 Concentration of cGMP in the blood plasma of control, L-NAME and DCLHb treated rats. Blood samples were collected 15 min after administration of vehicle (4 ml/kg iv, N = 6), DCLHb (400 mg/kg iv, N = 10), L-NAME (10 mg/kg iv, N =7) and L-NAME (10 mg/kg iv) plus DCLHb (400 mg/kg iv) (N = 7) treated rats. *Indicates significant difference compared to control group and +indicates significant difference compared to DCLHb group.

Effect of L-NAME pretreatment on DCLHb-induced changes in systemic hemodynamics

L-NAME (10 mg/kg iv) *per se* produced significant increase in blood pressure (51%) and total peripheral resistance (120%), and decrease in cardiac output (-19%) and stroke volume (-14%). DCLHb, in L-NAME pretreated rats, did not produce any further increase in blood pressure. However, DCLHb significantly decreased the cardiac output and stroke volume in L-NAME treated rats, when compared to values obtained after L-NAME pretreatment. The decrease in cardiac output ($P = 0.01$) and stroke volume ($P = 0.044$) were found to be significantly more marked in L-NAME treated group than control group. The increase in total peripheral resistance in L-NAME-treated rats was found to be significantly ($P = 0.04$) greater than control group. DCLHb did not produce any further increase in the total peripheral resistance in L-NAME pretreated animals (Fig. 3).

Effect of L-NAME pretreatment on DCLHb-induced changes in the regional blood flow and vascular resistance

L-NAME *per se*, did not produce any change in regional blood flow, however, an increase in vascular resistance was observed in the brain, GIT, liver, mesentery and pancreas, kidney and skin. DCLHb when administered to L-NAME treated rats produced a significant increase in blood flow to the heart (198%) and decrease in blood flow to the liver (-36%), mesentery and pancreas (-47%), kidneys (-48%) and musculoskeletal system (-56%). In L-NAME pretreated rats, DCLHb-induced effects on regional blood flow were found to be significantly attenuated in the GIT ($P = 0.04$), mesentery and pancreas ($P = 0.02$), kidneys ($P = 0.04$) and musculoskeletal system ($P = 0.004$) as compared to control group. DCLHb induced increase in blood flow to the heart was not affected by L-NAME pretreatment. L-NAME followed by DCLHb increased vascular resistance in the kidneys, spleen, mesentery and pancreas, whereas vascular resistance was found to be significantly decreased (-32%) in the heart. DCLHb induced effects on regional vascular resistance in L-NAME pretreated rats, were found to be significantly higher in the GIT ($P = 0.02$), liver ($P = 0.02$), mesentery and pancreas ($P = 0.005$), kidneys ($P = 0.04$), skin ($P = 0.02$), and musculoskeletal system ($P = 0.01$) as compared to control group (Fig. 4). DCLHb produces minimal decrease in vascular resistance in the heart, however, the decrease in vascular resistance is more marked in L-NAME pretreated rats (Figs. 1 and 4)

Effect of DCLHb on the cGMP concentration in the blood plasma

In control animals, concentration of cGMP in the circulating arterial blood plasma was found to be 7.43 ± 0.76 pmoles/ml ($N = 6$). Both L-NAME and DCLHb *per se*, produced a significant ($P < 0.0001$ and $P < 0.0001$, respectively) decrease in cGMP concentration in the circulating blood plasma. L-NAME followed by DCLHb administration significantly decreased cGMP levels when compared with either DCLHb ($P = 0.02$) or control ($P < 0.0001$) group (Fig. 5).

8.4 Discussion

DCLHb, prepared by αα crosslinking within the haemoglobin tetramer, possess biochemical stability and has greater intravascular retention time than unmodified SFHb (Hess *et al.*, 1989). It does not require cross-matching or typing prior to administration. DCLHb is devoid of white cells and other blood components which are known to release cytotoxic products. It does not interfere with coagulation cascade or the reticular endothelial system (Gulati *et al.*, 1994; Schultz *et al.*, 1993). Infusion of DCLHb was associated with an increase in mean arterial pressure (Gulati and Rebello, 1994; Hamilton *et al.*, 1992). The pressor effect of DCLHb has been shown to be self-limiting and was not due to volume load or oncotic pressure (Hamilton *et al.*, 1992). The results of present study indicate that the pressor effect of DCLHb was accompanied by an increase in total peripheral resistance and cardiac output. The increase in cardiac output was due to an increase in stroke volume while no significant change in heart rate was observed. DCLHb induced increase in cardiac output returned to baseline but total peripheral resistance increased significantly at 60 min after the infusion of DCLHb. Thus, in the initial phase the pressor effect of DCLHb was due to increases in both cardiac output and total peripheral resistance, while in the later phase the pressor effect is only due to an increase in total peripheral resistance.

Recent studies provide evidence that administration of DCLHb to rats produces significant regional circulatory changes (Sharma and Gulati, 1994; Sharma *et al.*, 1994). Unmodified SFHb has also been reported to produce a transient increase in blood pressure and blood flow to the heart and GIT (Ning *et al.*, 1992). However, significant differences have been observed in the regional circulatory effects of stroma-free haemoglobin and DCLHb (Gulati *et al.*, 1994). In the present study, DCLHb was found to produce an increase in blood flow to the heart, GIT, spleen, skin and mesentery and pancreas, while no change occurred in blood flow to the brain, kidneys, liver and musculoskeletal system. An increase in vascular resistance was observed in the kidneys, GIT, liver and spleen. Musculoskeletal system which receives about 55 % of the cardiac output showed a marked increase in vascular resistance. These observations are similar to those reported earlier, where it was found that an increase in blood flow to several organs occurs due to an increase in percent distribution of cardiac output to those organs and a decrease in the percent distribution of cardiac output to the musculoskeletal system (Sharma and Gulati, 1994).

It is well known that the iron in heme has an avid affinity for NO and that the NO released in several pathological situations is immediately inactivated by haemoglobin (Gibaldi, 1993). Several studies indicate that NO system is involved in the regulation of regional blood circulation. Increases in rat cortical blood flow due to stimulation of cerebrovascular parasympathetic nerves can be blocked by L-NAME treatment and restored by infusion of L-

arginine (Morita-Tsuzuki *et al.*, 1993). When administered to goats, L-NAME produced a significant decrease in cerebral blood flow and increase in blood pressure which could be reversed by L-arginine (Fernandez *et al.*, 1993). Inhibition of NO synthase activity by either L-NAME or N^G-Nitro-L-arginine produced constriction of small coronary arteries and arterioles (Jones *et al.*, 1993). Sigmon et al. found that systemic inhibition of NO synthesis leads to acute hypertension and increased peripheral vascular resistance (Sigmon *et al.*, 1993). The changes in vascular resistance are not evenly distributed to all vascular beds. They demonstrated that the NO system is a more important regulator of renal blood flow than of femoral blood flow. Hester et al. found that L-NAME attenuated the vasodilation of muscle arterioles in response to acetylcholine. The attenuation was more marked in first order and much less in second and third order blood vessels (Hester *et al.*, 1993). Since NO system is an important regulator of regional blood circulation, it could be possible that the regional circulatory effects of DCLHb are mediated through NO system. Studies were conducted to determine the role of NO in the mechanism of action of DCLHb.

The results of the present study indicate that L-arginine, a precursor of NO, blocks the cardiovascular effects DCLHb. The increase in blood pressure, cardiac output, stroke volume and total peripheral resistance induced by DCLHb were completely blocked by L-arginine. DCLHb induced increase in blood flow to the spleen and skin was completely blocked by L-arginine pretreatment. While the increase in blood flow induced by DCLHb to the heart was partially blocked and to GIT and mesentery and pancreas was not blocked by L-arginine pretreatment. DCLHb did not produce any significant decrease in blood flow to the musculoskeletal system, of normal rats but produced a significant decrease in L-arginine pretreated rats. It has been shown that the pressor effect of DCLHb can be reversed either by increasing the endogenous pool of NO by administering L-arginine, a substrate for NO synthase, or by administering nitroglycerine, an agent that liberated NO (Schultz *et al.*, 1993). The present study also shows that systemic hemodynamic and regional circulatory effects of haemoglobin based blood substitutes could be attenuated by increasing the generation of endogenous NO. The increased generation tends to compensate for the inactivation of NO by haemoglobin. It is interesting to note that systemic hemodynamic effects of DCLHb are completely blocked and most of the regional circulatory effects of DCLHb are also blocked by L-arginine. These studies provide evidence that NO system is, at least in part, involved in the cardiovascular effects of DCLHb.

Further studies were conducted using L-NAME, a NO synthase inhibitor. It was found L-NAME produced an increase in blood pressure and total peripheral resistance and a decrease in cardiac output and stroke volume. When DCLHb was administered to L-NAME treated rats no further increase in blood pressure or total peripheral resistance was observed but further decrease in cardiac output and stroke volume was produced. L-NAME did not affect blood flow

to various organs but produced a significant increase in vascular resistance in the brain, GIT, kidneys, liver, musculoskeletal system and skin. DCLHb when administered to L-NAME treated rats produced a significant increase in blood flow to the heart and decrease in blood flow to the liver, GIT, kidneys, liver, spleen, mesentery and pancreas, musculoskeletal system and skin. DCLHb produced a significant increase in vascular resistance in the GIT, kidneys, liver, spleen, mesentery and pancreas, musculoskeletal system and skin. Vascular resistance in the heart was significantly decreased by DCLHb in L-NAME rats. These observations suggest that L-NAME leads to a decrease in the generation of NO thereby reducing the dilator action of endogenous NO, thus producing an increase in vascular resistance and blood pressure. DCLHb due to NO scavenging action produces a further increase in regional vascular resistance and a decrease in blood flow to various organs. It appears that L-NAME has reduced NO levels to such an extent in some organs that not much was available for DCLHb to scavenge upon. Thus, in most of the organs no further regional circulatory effects were produced by DCLHb. The only organ, where DCLHb produced effect was the heart. It appears that either NO mechanisms are extremely potent in the heart as compared to other vascular beds or some other mechanism is also involved in the increase in blood flow to the heart by DCLHb.

The physiological and pharmacological actions of NO are all mediated by the activation of soluble guanylate cyclase and the consequent increase in the concentration of cyclic guanosine monophosphate (cGMP) (Moncada and Higgs, 1993). Studies were conducted to determine the concentration of cGMP in control, DCLHb, L-NAME and DCLHb plus L-NAME treated rats. It was found that both DCLHb and L-NAME significantly decreased the concentration of cGMP indicating that L-NAME has reduced the formation of NO, while DCLHb has removed the available NO leading to decrease in the activity of guanylate cyclase and consequent decrease in cGMP. It was interesting to note that when DCLHb was given in L-NAME pretreated rats a further decrease in cGMP concentration was observed indicating that although L-NAME decreased the formation of NO but whatever NO was available was scavenged by DCLHb.

Recent studies have shown that haemoglobin solutions may also act through other mechanisms. When administered to rats, DCLHb has been shown to produce a potentiation of the blood pressure effects of norepinephrine and phenylephrine (Gulati and Rebello, 1994). The pressor effect of DCLHb was attenuated by phosphoramidon, an endothelin converting enzyme inhibitor (Schultz *et al.*, 1993). It appears that besides NO, other mechanisms are also involved in the cardiovascular actions of haemoglobin based blood substitutes. However, this study provides clear evidence for the role of NO in systemic hemodynamics and the regional circulatory effects of DCLHb.

8.5 Acknowledgments

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Part 5

Role of endothelin mechanism in the cardiovascular effects of diaspirin crosslinked haemoglobin

Chapter 9

Effect of diaspirin crosslinked and stroma-reduced haemoglobin on mean arterial pressure and endothelin-1 concentration in rats

Summary

The effect of unmodified stroma reduced (SRHb) and modified diaspirin crosslinked (DCLHb™) haemoglobin solutions on the mean arterial pressure and endothelin-1 (ET-1) concentration in blood plasma and various tissues was studied. Infusion of DCLHb or SRHb increased mean arterial blood pressure by 96 % and 39 %, respectively. Heart rate was not significantly affected by DCLHb or SRHb. A significant increase ($P < 0.003$) in the ET-1 levels in blood plasma after DCLHb and SRHb infusion was observed. The increase in plasma ET-1 concentration was significantly more marked with SRHb (141 %) as compared to DCLHb (78 %) treated rats. The concentration of ET-1 in the heart and brain regions was not altered in DCLHb and SRHb treated rats as compared to control. However, ET-1 concentration was significantly increased in the thoracic aorta (151 %) and renal medulla (272 %) of DCLHb treated rats. SRHb treated rats also showed a significant increase in ET-1 concentration in the thoracic aorta (141 %) and renal medulla (429 %). The effect of SRHb on the renal medulla was found to be significantly greater than that of DCLHb. ET may be one of the factors responsible for the cardiovascular effects of haemoglobin solutions.

9.1 Introduction

Modified haemoglobin solutions are being developed as promising resuscitative solutions and offer several advantages as compared to alternatives (Bonhard, 1975; Kaplan and Murphy, 1975; Moss *et al.*, 1976; DeVenuto and Zegna, 1978). The effect of haemoglobin solutions on vascular activity has been of interest for decades. Haemoglobin solutions have been reported to produce a rapid and sustained increase in mean arterial pressure (Messmer *et al.*, 1977; Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). Several studies have been performed demonstrating cardiovascular actions of stroma free haemoglobin solutions. Purified stroma free haemoglobin solutions have been reported to produce vasoconstrictor activity in a variety of experimental models (Gilroy *et al.*, 1988; Vogel *et al.*, 1986). Recently, it has been reported that an unmodified human stroma-free haemoglobin solution produces an increase in the blood flow to the heart, brain, liver, gut and kidneys of the dog (Ning *et al.*, 1992).

DCLHb is an oxygen carrying protein prepared by cross-linking human haemoglobin between the α-subunits by means of a reaction with the diaspirin compound, bis(3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process used to produce DCLHb includes heat pasteurization of the solution (Estep *et al.*, 1989b; Estep *et al.*, 1989a) to inactivate any contaminating viruses and precipitate undesired proteins. DCLHb is

biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). DCLHb has also been shown to increase the mean arterial blood pressure (Przybelski *et al.*, 1990; Gulati and Rebello, 1994) and blood flow to several organs (Gulati and Sharma, 1994; Sharma and Gulati, 1994). The cardiovascular effects of DCLHb appear to be mediated through the nitric oxide (NO) (Schultz *et al.*, 1993; Katsuyama *et al.*, 1994), endothelin (ET) (Schultz *et al.*, 1993) and adrenergic systems (Gulati and Rebello, 1994). Although studies have implicated the role of ET, the effect of haemoglobin on ET has not been well studied (Schultz *et al.*, 1993). No study has been performed to determine the effect of haemoglobin solutions on the endogenous concentration of ET. The present study was conducted to determine the effect of DCLHb and SRHb on blood pressure and the endogenous concentration of ET-1 in blood plasma and various tissues.

9.2 Materials and Methods

Drugs and chemicals

Diaspirin cross-linked haemoglobin (DCLHb) was prepared and provided by Baxter Healthcare Corp., Round Lake, IL. Stroma-reduced haemoglobin (SRHb) was prepared at Baxter Healthcare Corp., Round Lake, IL by a method similar to that described earlier (Zager and Gamelin, 1989). Briefly, outdated human RBCs were separated by centrifugation and lysed by adding 2 vol of distilled water per 1 vol of RBCs followed by two freeze thawing procedures. The lysed cells were centrifuged for 15 min at 26,000 g. The supernatant containing the SRHb was collected and used for subsequent testing. The physicochemical properties of DCLHb were methaemoglobin 4.1 %, sodium 141 mEq/l, chloride 112 mEq/l and phospholipids 0.2 ppm, while those of SRHb were methaemoglobin 12.8 %, sodium 114 mEq/l, chloride 137 mEq/l and phospholipids >50 ppm. DCLHb (400 mg/kg; 10 %) and SRHb (400 mg/kg; 10 %) were administered in a volume of 4 ml/kg of solution through the left femoral vein. [^{125}I]Tyr^{13}-ET-1 (human, porcine) was purchased from DuPont NEN Research Products, Wilmington, DE. SEP-Columns (200 mg), rabbit anti-ET-1 (porcine, human) serum, normal rabbit serum and goat anti-rabbit IgG serum were purchased from Peninsula Laboratories Inc., Belmont, CA. The radioimmunoassay buffer consisted of 19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 0.05 M NaCl, 0.1 % bovine serum albumin, 0.1 % TritonX-100 and 0.01 % NaN_3.

Animals

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI), acclimatized to the environment, weighing 300-350 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing)

for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from the blood pressure. In order to keep the blood pO_2, pCO_2 and pH constant and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a Harvard Rodent Ventilator Model 683.

Collection of samples

In another group of rats the animals were sacrificed 15 min after administration of saline, DCLHb or SRHb. Arterial and venous blood samples were collected in plastic tubes containing ethylenediaminetetraacetate (1 mg/ml) and aprotinin (500 kIU/ml), centrifuged (15 min, 4 °C, 1000 x g) and the plasma was collected. Plasma was mixed with an equal volume of 20% acetic acid and centrifuged (15 min, 4°C, 1000 x g) and the supernatant was collected for subsequent assay. Tissue samples were collected in another group of rats which were anesthetized and circulating blood was removed following perfusion with saline. Brain regions (hypothalamus, pons and medulla, cerebral cortex, pituitary and midbrain), heart, kidneys and thoracic aorta were dissected out and weighed. Tissues were homogenized in 10% acetic acid (10 ml per gram wet weight) using a Polytron homogenizer (setting 5 for 20 sec). The homogenate was centrifuged at 49,000 x g for 15 min at 4°C, and the supernatant was collected for subsequent assay.

Determination of ET-1 concentration by radioimmunoassay

The radioimmunoassay for ET-1 was carried out as described earlier (Singh *et al.*, 1994). All reagents were kept in ice and all procedures were performed at 4°C. The SEP-columns were activated by successively washing with 3 ml methanol, 2 ml water and 2 ml of 10% acetic acid solution. The samples, collected as described earlier, were slowly applied to the column and then allowed to flow through the column at the rate determined by gravity. The columns were washed with 2 ml of 10% acetic acid followed by 3 ml of ethyl acetate. ET was eluted with 1.5 ml of elution buffer (1 : 4; 0.05 M ammonium bicarbonate solution : methanol) into polypropylene tubes. The eluate was evaporated to dryness under a gentle stream of nitrogen gas. The recovery was found to be 86% by this method. The radioimmunoassay was performed using antibodies specific for ET-1 (Peninsula Lab. Inc, Belmont, CA). This antibody has only 7% cross reactivity with ET-2 or ET-3. The incubation mixture for the radioimmunoassay consisted of 100 μl of standards or samples and 100 μl of rabbit anti-ET-1 serum. The tubes were vortexed and incubated overnight at 4°C. The next day, [^{125}I] ET-1 (100 μl) was added and the mixture vortexed and incubated for 24 hours. On day 3, goat anti-rabbit IgG serum (100 μl) and normal rabbit serum (100 μl) were added to all the tubes,

vortexed and incubated for 2 h at room temperature. Radioimmunoassay buffer (500 μl) was added, the tubes were vortexed and then centrifuged at 1,700 g for 20 min. The supernatant was carefully aspirated and the radioactivity in the pellet was determined using Packard Gamma Counter (Model Cobra 5005). The concentration of ET-1 was determined from the standard plots of % total (B)/total-nonspecific (B_o) bindings (B/B_o) versus log dose (pg) and expressed as pg/ml in plasma and pg/g wet weight in tissues.

Statistics

All data are presented as mean values ± SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Duncan's test or Scheffe's S-test. A value of $P < 0.05$ was considered to be significant.

9.3 Results

Effect of DCLHb and SRHb on blood pressure and heart rate

DCLHb (400 mg/kg, i.v.) produced a significant increase ($P = 0.0001$) in mean arterial pressure. SRHb (400 mg/kg, i.v.) also produced a significant ($P = 0.0215$) pressor effect (Fig. 1). The increase in arterial pressure was immediate and lasted for more than 60 min following infusion of both haemoglobin solutions. The pressor effect of DCLHb was significantly ($P = 0.02$) more marked than that produced by SRHb. DCLHb and SRHb did not affect the heart rate. The infusion of an equal volume of saline did not produce any significant effect on mean arterial pressure or heart rate.

Effect of DCLHb and SRHb on ET-1 concentrations in blood plasma

Endogenous ET-1 like immunoreactivity was found to be similar in both the venous and arterial blood plasma samples of control (untreated) rats. Administration of both haemoglobin solutions significantly increased the concentration of ET-1 like immunoreactivity in blood plasma obtained from either the arterial ($P = 0.01$) or venous ($P = 0.01$) side of the circulation when compared with saline treated rats. The concentration of ET-1 like immunoreactivity was found to be significantly greater in SRHb treated rats, in both arterial ($P = 0.02$) and venous ($P = 0.04$) blood plasma samples as compared to DCLHb treated rats (Fig. 2).

Effect of DCLHb and SRHb on ET-1 concentrations in brain regions

Administration of haemoglobin solutions did not affect the concentration of ET-1 like immunoreactivity in any of the brain regions (hypothalamus, cerebral cortex, midbrain, pons and medulla) studied (Fig. 3).

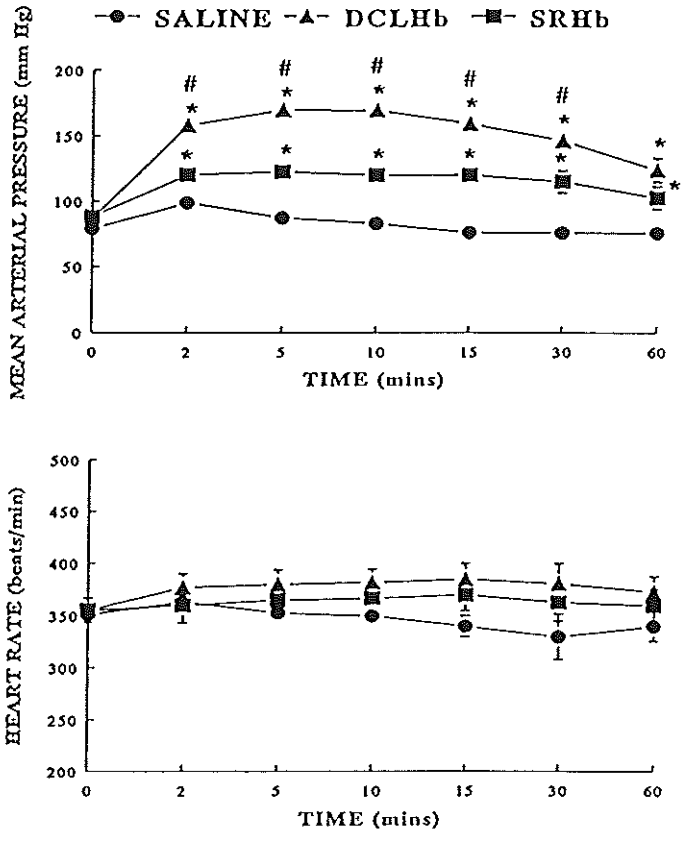

Fig. 1 The effect of saline (4 ml/kg, i.v.; N=7), DCLHb (400 mg/kg, i.v.; N=6) and SRHb (400 mg/kg, i.v.; N=6) on the mean arterial pressure (mmHg) and heart rate (beats/min) at 0 min, 2, 5, 10, 15, 30 and 60 min after administration to rats. * indicates significant difference as compared to control (saline) and # indicates significant difference as compared to SRHb treated rats.

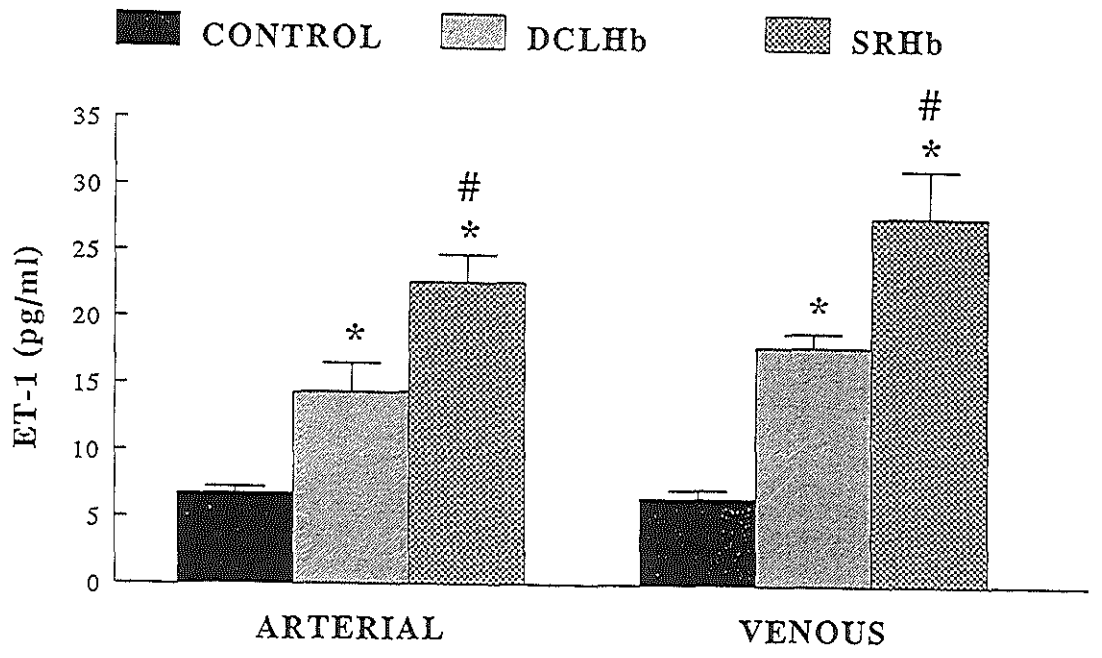

Fig. 2 The effect of saline (4 ml/kg, i.v.; N=7), DCLHb (400 mg/kg, i.v.; N=5) and SRHb (400 mg/kg, i.v.; N=7) on the ET-1 like immunoreactivity in arterial and venous blood plasma of rats. * indicates significant difference as compared to control (saline) and # indicates significant difference as compared to DCLHb treated rats.

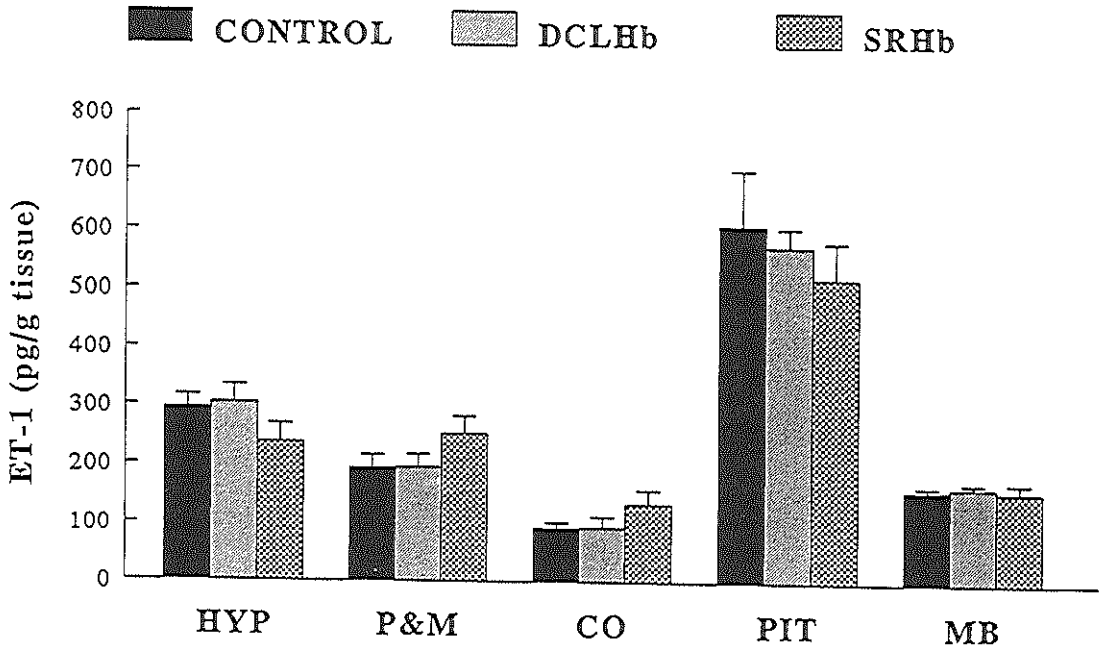

Fig. 3 The effect of saline (4 ml/kg, i.v.; N=4), DCLHb (400 mg/kg, i.v.; N=4) and SRHb (400 mg/kg, i.v.; N=4) on the ET-1 like immunoreactivity in the brain regions (HYP = hypothalamus, P&M = pons and medulla, CO = cerebral cortex, PIT = pituitary and MB = midbrain) of rats.

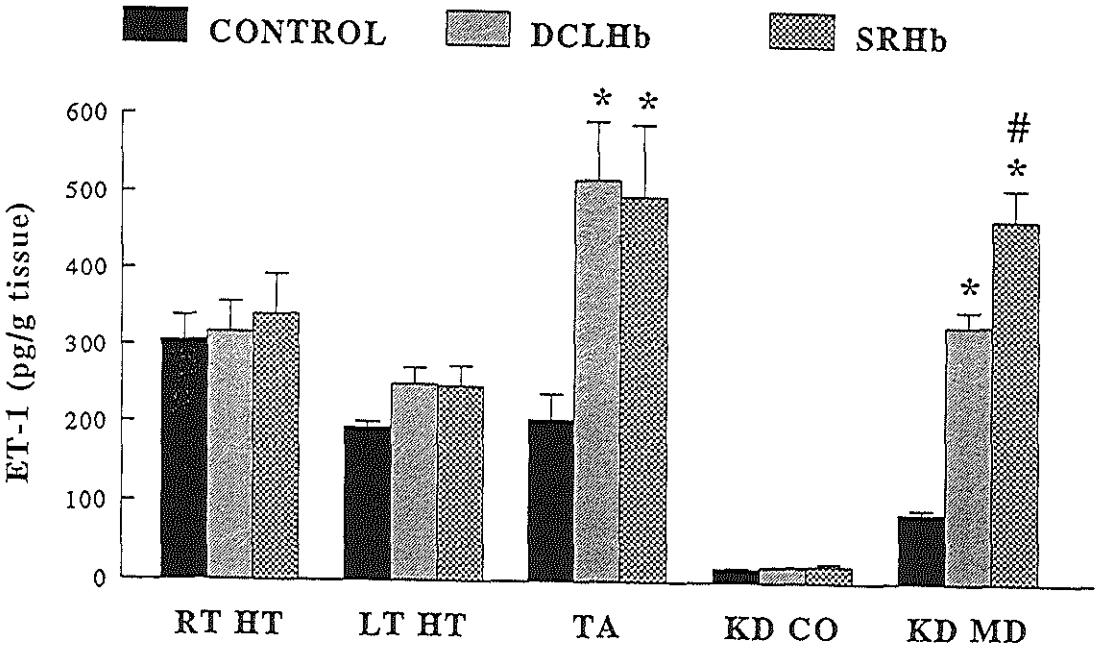

Fig. 4 The effect of saline (4 ml/kg, i.v.; N=5), DCLHb (400 mg/kg, i.v.; N=5) and SRHb (400 mg/kg, i.v.; N=5) on the ET-1 like immunoreactivity in the peripheral regions (RTHT = right heart, LTHT = left heart, TA = thoracic aorta, KDCO = renal cortex and KDMD = renal medulla) of rats. * indicates significant difference as compared to control (saline) and # indicates significant difference as compared to DCLHb treated rats.

Effect of DCLHb and SRHb on ET-1 concentrations in peripheral regions

Infusion of haemoglobin solutions significantly increased the concentration of ET-1 like immunoreactivity in the thoracic aorta of DCLHb ($P = 0.03$) and SRHb ($P = 0.04$) treated rats as compared to control. There was no change in ET-1 concentration in the heart and renal cortex, however, there was a significant increase in ET-1 like immunoreactivity in the renal medulla of DCLHb ($P = 0.0002$) and SRHb ($P = 0.0001$) treated rats as compared to control. The concentration of ET-1 like immunoreactivity was significantly greater in the renal medulla of SRHb treated rats ($P = 0.016$) as compared to DCLHb treated rats (Fig. 4).

9.4 Discussion

Early attempts at preparing haemoglobin based blood substitutes involved hypotonic lysis of human red blood cells followed by crude separation of haemoglobin from insoluble red cell membranes, but these haemoglobin solutions produced significant toxicity. Elimination of red cell stromal contaminants from haemoglobin solution by gentle lysis, centrifugation and filtration resulted in haemoglobin preparations that did not produce significant toxicity in dogs or primates (Rabiner *et al.*, 1967). Nevertheless the apparently stroma-free haemoglobin solutions produced significant adverse effects in human volunteers (Savitsky *et al.*, 1978) which were attributed to the presence of low levels of impurities (Bunn, 1993). Most of the previous studies, irrespective of the stromal contents, have described the haemoglobin solutions as stroma-free haemoglobin or purified stroma-free haemoglobin or highly purified stroma-free haemoglobin. In the present study we have denoted as stroma-reduced haemoglobin (SRHb) a haemoglobin preparation in which the stromal contents have been partially removed (Zager and Gamelin, 1989) from the soluble haemoglobin. The term stroma-free should ideally mean free of any stromal contents. DCLHb is not only a highly purified, but also chemically modified haemoglobin preparation. The phospholipid content which is a useful indicator of red cell membrane components (Biessels *et al.*, 1992) was found to be more than 50 ppm in SRHb, but in the order of 0.2 ppm in DCLHb.

Haemoglobin based blood substitutes have been reported to produce marked hemodynamic changes (Vogel *et al.*, 1986). DCLHb, a promising resuscitative solution (Przybelski *et al.*, 1991; Malcolm *et al.*, 1992) exhibits a pressor effect in the rat (Przybelski *et al.*, 1990; Gulati and Rebello, 1994) similar to that reported for other haemoglobin solutions (Rabinovici *et al.*, 1989; Waschke *et al.*, 1993; Kida *et al.*, 1991). Intravenous injection of ET-1 into animals causes an initial decrease in blood pressure which is followed by a prolonged pressor response (Inoue *et al.*, 1989) both in anesthetized and chemically denervated rats and in conscious rats (Miyauchi *et al.*, 1989; Rohmeiss *et al.*, 1990; King *et al.*, 1990). ET participates in the

maintenance of blood flow of peripheral small resistance vessels and has been implicated in the regulation of blood circulation (Masaki, 1994). ET acts on its receptors to produce biological actions. It is well known that ET receptors have a wide-spread distribution (Gulati and Srimal, 1992) and that at least two types of ET receptors exist. ET_A receptors have high affinity for ET-1 as compared to ET-3 (Arai *et al.*, 1990) and ET_B receptors have equal affinity for ET-1 and ET-3 (Sakurai *et al.*, 1990). ET_A receptors are mainly responsible for the vasoconstrictor effect of ET-1 and ET_B receptors have been shown to release NO, and thus, are responsible for the vasodilator action of ET-1 (de Nucci *et al.*, 1988). It is possible that haemoglobin solutions release ET-1 which could be responsible for their cardiovascular effects. We studied the hemodynamic and ET-1 immunoreactivity changes induced by unmodified, partially purified (SRHb) and modified, purified (DCLHb) haemoglobin solutions in blood plasma, brain and peripheral regions.

High levels of ET-1 like immunoreactivity were observed both in venous and arterial blood plasma of DCLHb and SRHb treated rats. This observation suggests that the hemodynamic alterations produced by DCLHb and SRHb infusion could be due to increased levels of ET-1 in the blood circulation. SRHb treated rats exhibited higher levels of plasma ET-1 like immunoreactivity in comparison to DCLHb treated rats, suggesting that SRHb produces a more marked effect on ET generation or release. If plasma ET levels are responsible for the cardiovascular effects of haemoglobin solutions, it would be expected that the pressor effect of SRHb would be more marked than DCLHb. However, the present study clearly shows that the pressor effect of DCLHb is more marked than that of SRHb. It is not known whether the excess release of ET-1 by SRHb contributes more towards the action of ET-1 on ET_B receptors causing excessive release of NO, thus leading to the reduced pressor effect.

The differences in the ability of SRHb and DCLHb to initiate an inflammatory reaction have been demonstrated (Burhop *et al.*, 1992). Infusion of SRHb in sheep has been found to increase the plasma concentrations of C3a and thromboxane B_2, while DCLHb did not elicit any increase in C3a and thromboxane B_2 (Burhop *et al.*, 1992). The presence of phospholipids in the SRHb solution could be responsible for the differences in observed responses. Phosphoramidon, an inhibitor of pro-ET conversion to ET, attenuated the pressor effect of DCLHb (Schultz *et al.*, 1993). It appears that haemoglobin solutions enhance either the conversion of pro-ET to ET or the release of ET. Oxyhaemoglobin has been found to increase the production of ET-1 by endothelial cells in culture (Ohlstein and Storer, 1992; Cocks *et al.*, 1991). Although significant changes in blood plasma ET-1 concentration were observed following administration of SRHb and DCLHb, only a few tissues showed an alteration in ET-1 concentration. In brain regions, there was no significant change observed in ET-1 like immunoreactivity after DCLHb or SRHb infusion, probably because haemoglobin and ET-1

do not cross the blood brain barrier. No change in ET-1 concentration was observed in the heart and renal cortex. However, in the thoracic aorta and renal medulla, a significant increase in ET-1 concentration was produced by DCLHb and SRHb.

High levels of ET like immunoreactivity have been detected in red blood cells and it was found that ET like immunoreactivity coeluted with haemoglobin (Hemsen and Lundberg, 1992). In the present study an extraction procedure was performed using SEP-columns which have been shown to reduce the coelution of ET like immunoreactivity with haemoglobin (Hemsen and Lundberg, 1992). Since ET-1 levels were significantly increased in the tissues (thoracic aorta and renal medulla) and the interference of haemoglobin solutions in tissue homogenate preparation is not possible, therefore the increase in ET-1 concentrations following administration of SRHb or DCLHb appear to be due to either increased generation and/or release of ET. It is not clear whether the increase in the concentration of ET is likely to contribute to the pressor effect of SRHb or DCLHb. Intravenous injection of ET into animals causes an initial decrease in blood pressure which is followed by a prolonged pressor response (Inoue *et al.*, 1989) both in anesthetized and chemically denervated rats and conscious rats (Miyauchi *et al.*, 1989; Rohmeiss *et al.*, 1990; King *et al.*, 1990). On the other hand, a low dose of ET produces hypotension and vasodilatation of isolated perfused arteries *in vitro* (Lippton *et al.*, 1988; Kitazumi *et al.*, 1990). ET-1 produces markedly different effects on blood circulation in different regions (Wright and Fozard, 1988; Le Monnier de Gouville *et al.*, 1990; Gardiner *et al.*, 1990; Minkes and Kadowitz, 1989). For example, after ET-1 infusion blood flow decreased in splanchnic areas, while it increased in the skeletal muscles. ET-1 can therefore cause either vasodilatation or vasoconstriction depending on the region (Inoue *et al.*, 1989; Gardiner *et al.*, 1990). Disruption of the ET-1 gene by homologous recombination to generate mice deficient in ET-1 resulted in elevated blood pressure in these mice. The response of blood pressure to exogenous ET-1 was not different between $ET\text{-}1^{+/-}$ heterozygous and $ET\text{-}1^{+/+}$ wild-type mice indicating that blood pressure elevation in ET-1 deficient mice is not due to hypersensitivity to ET-1 (Kurihara *et al.*, 1994). Due to conflicting reports, the exact role of ET-1 in the regulation of blood pressure is not clear. It is difficult to speculate on the exact role of ET-1 in the cardiovascular effects of haemoglobin solutions, but it appears that the pharmacological effects of haemoglobin solutions are also influenced by the process of preparation and purification of haemoglobin solutions.

The results of the present study clearly demonstrate that haemoglobin solutions, DCLHb and SRHb, lead to an increase in ET-1 like immunoreactivity in blood plasma, thoracic aorta and renal medulla. It is speculated that the interaction of haemoglobin solutions with the ET system contributes to some of the pharmacological actions of haemoglobin solutions.

9.5 Acknowledgments

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Chapter 10

Role of endothelin converting enzyme in the systemic hemodynamics and regional circulatory effects of proendothelin-1 [1-38] and diaspirin crosslinked haemoglobin in rats

Summary

Diaspirin crosslinked haemoglobin (DCLHb™; Baxter Healthcare Corp.) is a promising haemoglobin based oxygen carrying resuscitative solution. DCLHb (400 mg/kg, i.v.) produces significant cardiovascular effects when administered to conscious or anesthetized rats along with an increase in plasma endothelin-1 (ET-1) levels. Present studies were performed to determine whether the cardiovascular effects of DCLHb are due to an increase in the conversion of proET-1 to ET-1 by endothelin converting enzyme (ECE). The regional circulatory and systemic hemodynamic effects of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) were determined using a radioactive microsphere technique in control and phosphoramidon (endothelin converting enzyme inhibitor) pretreated rats. Administration of proET-1 produced an immediate increase in mean arterial pressure (52%) and total peripheral resistance (55%), stroke volume and cardiac output were not affected in the initial phase but were decreased subsequently. Heart rate was not affected following administration of proET-1. A significant increase in blood flow to the heart (39%), brain (46%), kidneys (74%), portal system (40%), and gastrointestinal tract (42%, GIT) was also observed following administration of proET-1. Vascular resistance was found to be significantly increased in the mesentery and pancreas (168%) and musculo-skeletal system (147%), and decreased in the kidneys (-11 %) following administration of proET-1. Phosphoramidon (4 mg/kg, i.v.) pretreatment attenuated the increase in mean arterial pressure and total peripheral resistance induced by proET-1. Phosphoramidon pretreatment significantly attenuated the proET-1 induced increase in blood flow to the heart, brain, kidneys, portal system and GIT. The increase in vascular resistance induced by proET-1 in the mesentery and pancreas and musculo-skeletal system was also attenuated by phosphoramidon. DCLHb increased mean arterial pressure (63%) and total peripheral resistance (54%) without affecting heart rate. DCLHb increased blood flow to the heart (95%), GIT (45%), portal system (43%) and skin (79%) and increased vascular resistance in the musculo-skeletal system (58 %). In phosphoramidon treated rats, DCLHb increased mean arterial pressure (99%), heart rate (25%), cardiac output (37%), and total peripheral resistance (60%). DCLHb increased blood flow to the heart (104%), brain (66%), kidneys (49%), GIT (59%), portal system (63%) and skin (100%) when administered to phosphoramidon treated rats. Phosphoramidon did not attenuate any of the DCLHb induced cardiovascular effects. It is concluded that proET-1 increases blood flow to various organs and phosphoramidon, an ET converting enzyme inhibitor, could block the proET-1 induced increases in regional blood flow. DCLHb induced increase in blood flow to several organs could not be blocked by phosphoramidon indicating that the cardiovascular effects of DCLHb are not due to an increase in the conversion of proET-1 to ET-1.

10.1 Introduction

Modified haemoglobin based solutions are being developed as potential resuscitative solutions and several advantages of these solutions over non-haemoglobin based resuscitative solutions have been reported (Kaplan and Murphy, 1975; Moss *et al.*, 1976). DCLHb is a modified haemoglobin solution derived from human erythrocytes. It is prepared by cross-linking haemoglobin between the α-subunits, within the haemoglobin tetramer, by means of a reaction with the diaspirin compound, bis(3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). It is purified by heat pasteurization to inactivate any contaminating viruses and precipitate any undesirable proteins (Estep *et al.*, 1989). It possesses biochemical stability and exhibits a greater intravascular retention time than unmodified haemoglobin. (Hess *et al.*, 1989; Estep *et al.*, 1991) It is not degraded in the blood stream nor does it accumulate in the tissues, but it is rapidly catabolized in tissues to low molecular weight compounds and eliminated through the urine and feces (Estep *et al.*, 1991) DCLHb does not require cross-matching or typing prior to administration, is less viscous than whole blood, and may be better able to carry oxygen through narrowed vessels to ischemic tissues due to the smaller size of the haemoglobin molecule relative to the erythrocytes. DCLHb is devoid of white cells and other blood components which are known to contribute to ischemic tissue injury by releasing cytotoxic products. It does not interfere with the coagulation cascade, or the reticular endothelial system. In situations simulating clinical settings, DCLHb was found to be effective as a resuscitative fluid following hemorrhage in rats, even at volumes one-half that of whole blood (Przybelski *et al.*, 1991). It was found that DCLHb maintains cardiac and renal functions after partial or complete exchange transfusions in swine (Hess *et al.*, 1989).

Earlier studies have shown that DCLHb infusion produces an increase in MAP in anaesthetized (Gulati *et al.*, 1994; Gulati and Rebello, 1994) and conscious (Schultz *et al.*, 1993) rats. This increase in MAP appears to be due to pharmacological properties of DCLHb and is not due to volume load or oncotic pressure (Hamilton *et al.*, 1992). DCLHb has also been found to produce a significant increase in blood flow to several organs such as the heart, GIT, portal system and skin. The increase in blood flow to these organs is accompanied by an increase in vascular resistance in the musculoskeletal system (Sharma and Gulati, 1994). Studies have been carried out to determine the pathogenesis of DCLHb induced cardiovascular effects. It has been established that endothelial cells play an important role in the regulation of vascular tone by producing vasoactive substances such as nitric oxide, which produce relaxation of the blood vessels (Vanhoutte *et al.*, 1986). L-arginine, the substrate for nitric oxide synthesis, and nitroglycerine, a nitric oxide donor, significantly reduced the DCLHb induced increase in MAP when infused 15 min after DCLHb administration (Schultz *et al.*, 1993). Another vasoactive substance produced by endothelial cells is ET-1, a potent vasoconstrictor peptide (Yanagisawa

et al., 1988). We have observed that plasma ET-1 concentration increases after DCLHb administration and the cardiovascular effects of DCLHb could be blocked by pretreatment with an ET-1 receptor antagonist, BQ-123 (Gulati *et al.*, 1996). It could be possible that DCLHb increases the conversion of proET-1 to ET-1 which could be responsible for the cardiovascular actions of DCLHb. This hypothesis is supported by the observation that oxyhaemoglobin increases the production of ET-1 by endothelial cells in culture (Cocks *et al.*, 1991; Ohlstein and Storer, 1992). It has also been found that the pressor effect of DCLHb could be attenuated by pretreatment with an ECE inhibitor, phosphoramidon (Schultz *et al.*, 1993). No study has yet been performed to determine the effect of an ECE inhibitor on DCLHb induced regional circulatory changes. Since ET-1 is known to act in a paracrine manner (Sharma and Gulati, 1995) and is a potent regulator of vascular tone, it was thought that studying the role of ECE on the regional circulatory effect of DCLHb, rather than on MAP, would be more appropriate.

The present studies were conducted to determine whether systemic hemodynamic and regional circulatory effects of DCLHb and proET-1 could be attenuated by phosphoramidon.

10.2 Materials and Methods

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, Wis.) weighing 325-350 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, i.p.). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant, and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a rodent ventilator (model 683, Harvard Apparatus Inc., South Natick, Mass.). Arterial blood pO_2, pCO_2 and pH were measured using a pH/blood gas analyzer (ABL330 Radiometer, Copenhagen, Denmark). The carotid artery of the right side was exposed and a PE 50 tubing was guided through the common carotid artery into the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P23 DC pressure transducer (Grass Instrument Co., Quincy, Mass.). When the cannula reached the ventricle, the diastolic pressure dropped to zero. The femoral artery of the right side was cannulated and connected to a withdrawal pump (Harvard Model 22).

Total haemoglobin, percent oxyhaemoglobin, carboxyhaemoglobin and methaemoglobin were measured using a hemoximeter (IL482 Co-oximeter system, Instrumentation Laboratory,

Lexington, Mass, USA). Total oxygen content was calculated as [Hb x 1.39 x $\%O_2Hb$] and oxygen capacity was calculated as [THb x 1.39 x (%RHb+$\%O_2Hb$)].

Determination of systemic hemodynamics and regional circulation

At each measurement, a thoroughly mixed suspension of approximately 100,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, Mass.) in 0.2 ml saline were injected into the left ventricle and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all the tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: lungs, heart, liver, stomach, small intestine, caecum, large intestine, mesentery and pancreas, spleen, left kidney, right kidney, left cerebral hemisphere, right cerebral hemisphere, midbrain, cerebellum, brain stem, skin and the rest of the body consisting of muscles and bones. The radioactivity in the standards, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter (Packard Instrument Co., Downers Grove, Ill.) with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output, (2) stroke volume, (3) total peripheral resistance, (4) regional blood flow and (5) regional vascular resistance. The data were calculated using the computer programs described earlier (Saxena *et al.*, 1980). The animals were divided into five groups. The effect of DCLHb (400 mg/kg, i.v.) on the systemic hemodynamics and regional circulation was studied in normal (group 1, N=9) and phosphoramidon (4 mg/kg, i.v.; group 2, N=8) treated rats. The effect of proET-1 (20 μg/kg, i.v.) was studied in normal (group 3, N=8) and phosphoramidon (4 mg/kg, i.v.; group 4, N=8) treated rats. Saline was administered in the fifth group (N=7) and did not produce any significant effect on cardiovascular parameters (data not presented). DCLHb, proET-1 and saline were administered in equal volumes. Phosphoramidon was administered 30 sec prior to either proET-1 or DCLHb. The dose of DCLHb was selected on the basis of studies conducted previously (Gulati and Rebello, 1994; Hamilton *et al.*, 1992) which demonstrated that infusion of this dose of DCLHb resulted in a near maximal pressor response The doses of proET-1 and phosphoramidon were also selected based on a previous study (Schultz *et al.*, 1993).

Statistics

All data are presented as the mean values ± 1 SEM. Mean arterial pressure (MAP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by two way analysis of variance followed by Duncan's test. A level of $P < 0.05$ was considered significant.

10.3 Results

Blood gases were not affected by either saline or DCLHb. The baseline values of pH, pO_2 and pCO_2 were found to be 7.4 ± 0.08, 146 ± 36 mmHg and 29.8 ± 7.8 mmHg, respectively. Following treatment with saline the pH, pO_2 and pCO_2 values were found to be 7.4 ± 1.10, 157 ± 7 mmHg and 27.6 ± 3.4 mmHg, respectively. Following treatment with DCLHb the pH, pO_2 and pCO_2 values were found to be 7.4 ± 0.05, 141 ± 8 mmHg and 28.4 ± 1.6 mmHg, respectively. The baseline hemoximeter values were: total haemoglobin = 14.3 ± 1.1 g/dl, oxyhaemoglobin = 95.9 ± 1.2 %, carboxyhaemoglobin = 0.9 ± 0.7 %, methaemoglobin = 1.2 ± 0.2 %, deoxyhaemoglobin = 2.1 ± 1.4 %, oxygen content = 19.0 ± 1.3 vol%, oxygen saturation = 97.9 ± 1.4 % and oxygen capacity = 19.5 ± 1.5 vol%. Administration of saline or DCLHb did not change these values significantly.

Effect of phosphoramidon on proET-1 induced changes in systemic hemodynamics

Administration of proET-1 (20 μg/kg, i.v.) produced an immediate increase in MAP [F(3,28) =7.73; p = 0.0007] and TPR [F = 2.07; p = 0.04] which lasted for 60 min in control (untreated) rats. No immediate change in CO and SV was observed, but a delayed decrease in CO and SV was observed after the administration of proET-1 to control rats. ProET-1 had no effect on HR in control rats (Table 1). Phosphoramidon (4 mg/kg, i.v.) *per se* did not produce any significant change in systemic hemodynamics in rats (data not shown). In phosphoramidon (4 mg/kg, i.v.) pretreated rats, the effect of proET-1 on the increase in MAP and TPR was significantly attenuated as compared to control rats. However, the delayed decrease in SV and CO induced by proET-1 was not affected by pretreatment with phosphoramidon (Table 1). HR was found to be significantly increased in phosphoramidon treated as compared to control rats (Table 1).

Table 1 Effect of proET-1 (20 μg/kg, i.v.) on the percent change in systemic hemodynamic parameters of control (vehicle, N = 8) and phosphoramidon (4 mg/kg, i.v., 30 sec before the administration of proET-1; N = 8) pretreated rats.

Parameter	Baseline	15 min	30 min	60 min
		Percent Change		
Heart rate (beats/min)				
proET-1	372 ± 30	0.3 ± 5.3	1.5 ± 5.7	4.7 ± 3.4
Phos. + proET-1	336 ± 18	11.7 ± 5.2	17.2 ± 5.5*	17.5 ± 3.7*#
Mean BP (mmHg)				
proET-1	63 ± 4	51.7 ± 5.8*	39.9 ± 7.8*	16.3 ± 7.6
Phos. + proET-1	65 ± 3	18.6 ± 7.5*#	34.6 ± 6.1*	14.1 ± 3.8*
Cardiac output (ml/min)				
proET-1	87 ± 6	0.3 ± 6.7	-9.7 ± 4.8	-13.7 ± 5.4*
Phos. + proET-1	77 ± 4	-0.3 ± 4.6	-0.7 ± 5.7	-16.8 ± 5.6*
Stroke Volume (ml)				
proET-1	0.25 ± 0.02	0.4 ± 5.6	-9.7 ± 5.9	-16.6 ± 6.6*
Phos. + proET-1	0.22 ± 0.01	-8.7 ± 7.1	-13.8 ± 6.6	-29.1 ± 4.1*
TPR (mmHg/l/min)				
proET-1	719 ± 58	55.3 ± 10.3*	58.3 ± 12.6*	39.4 ± 13.8*
Phos. + proET-1	830 ± 62	16.6 ± 8.8#	39.5 ± 11.4*	40.6 ± 8.8*

*$p < 0.05$ as compared to baseline and #$p < 0.05$ as compared to control (proET-1).

Table 2 Effect of DCLHb (400 mg/kg, i.v.) on the percent change in systemic hemodynamic parameters of control (vehicle, N = 9) and phosphoramidon (4 mg/kg, i.v., 30 sec before the administration of DCLHb; N = 8) pretreated rats.

Parameter	Baseline	15 min	30 min	60 min
		Percent Change		
Heart rate (beats/min)				
DCLHb	397 ± 16	1.3 ± 1.8	1.9 ± 1.7	1.2 ± 1.4
Phos. + DCLHb	324 ± 25	19.0 ± 4.3*#	20.2 ± 5.1*#	24.7 ± 6.7*#
Mean BP (mmHg)				
DCLHb	74 ± 6	63.1 ± 4.1*	53.7 ± 8.0*	35.9 ± 8.9*
Phos. + DCLHb	63 ± 3	98.6 ± 8.8*#	81.6 ± 10.9*#	72.0 ± 15.7*
Cardiac output (ml/min)				
DCLHb	96 ± 6	19.4 ± 5.2	34.5 ± 16.9	-9.8 ± 3.5
Phos. + DCLHb	87 ± 5	36.7 ± 5.4*	18.0 ± 11.6	10.8 ± 11.4
Stroke Volume (ml)				
DCLHb	0.24± 0.01	17.6 ± 4.8	32.8 ± 17.9	-10.8 ± 3.5
Phos. + DCLHb	0.28 ± 0.03	16.1 ± 6.9	-2.4 ± 7.5	-11.7 ± 6.2
TPR (mmHg/l/min)				
DCLHb	803 ± 92	37.9 ± 8.6*	22.9 ± 11.4*	54.0 ± 6.6*
Phos. + DCLHb	741 ± 47	46.1 ± 6.1*	59.8 ± 11.8*	60.4 ± 14.7*

*$p < 0.05$ as compared to baseline and #$p < 0.05$ as compared to control (DCLHb).

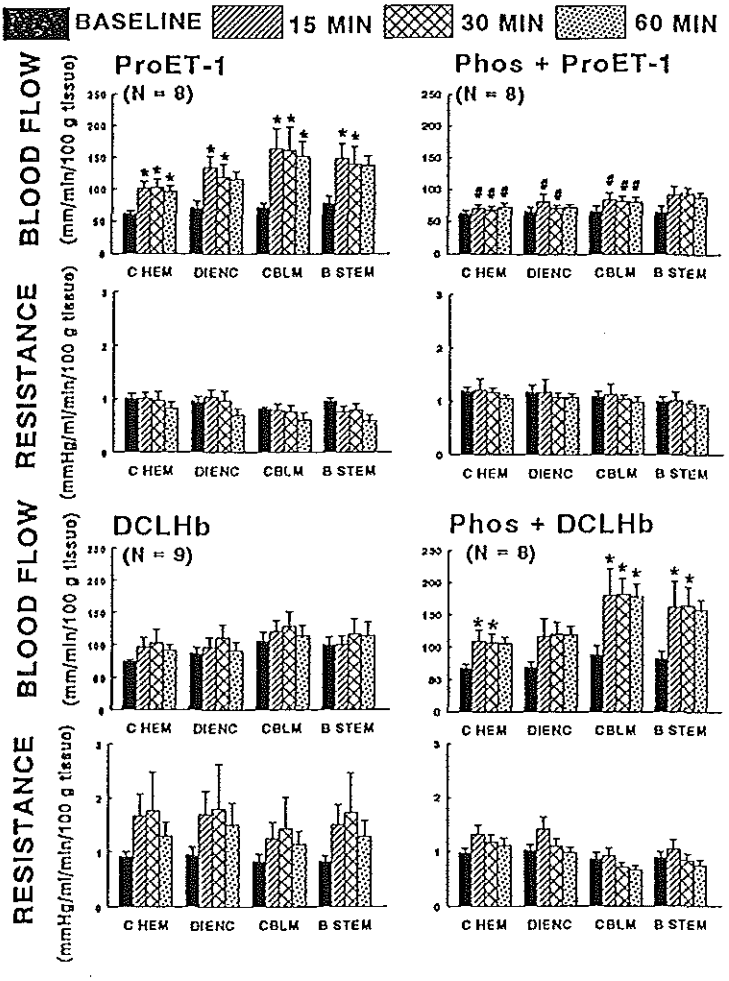

Fig. 1 The effect of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the cerebral hemispheres (C HEM), diencephalon (DIENC), cerebellum (CBLM) and brain stem (B STEM) before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated) and phosphoramidon (4 mg/kg, i.v.; 30 sec before the administration of proET-1 or DCLHb) treated rats. * Indicates significant difference as compared to baseline and # indicates significant difference as compared to control group.

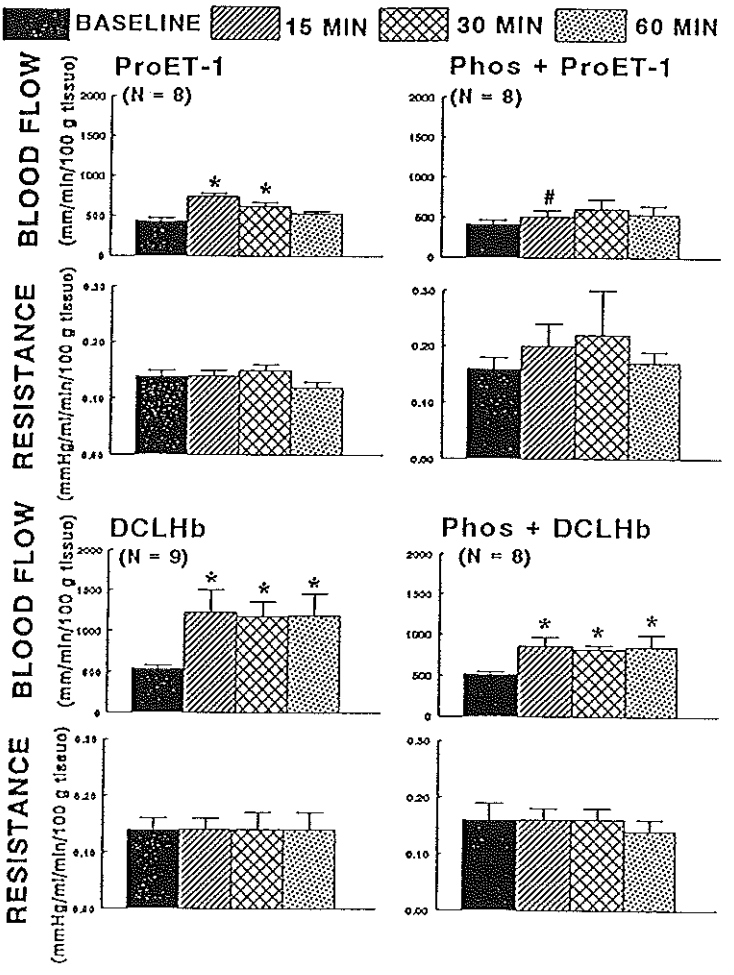

Fig. 2 The effect of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the heart before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated) and phosphoramidon (4 mg/kg, i.v.; 30 sec before the administration of proET-1 or DCLHb) treated rats. * Indicates significant difference as compared to baseline and # indicates significant difference as compared to control group.

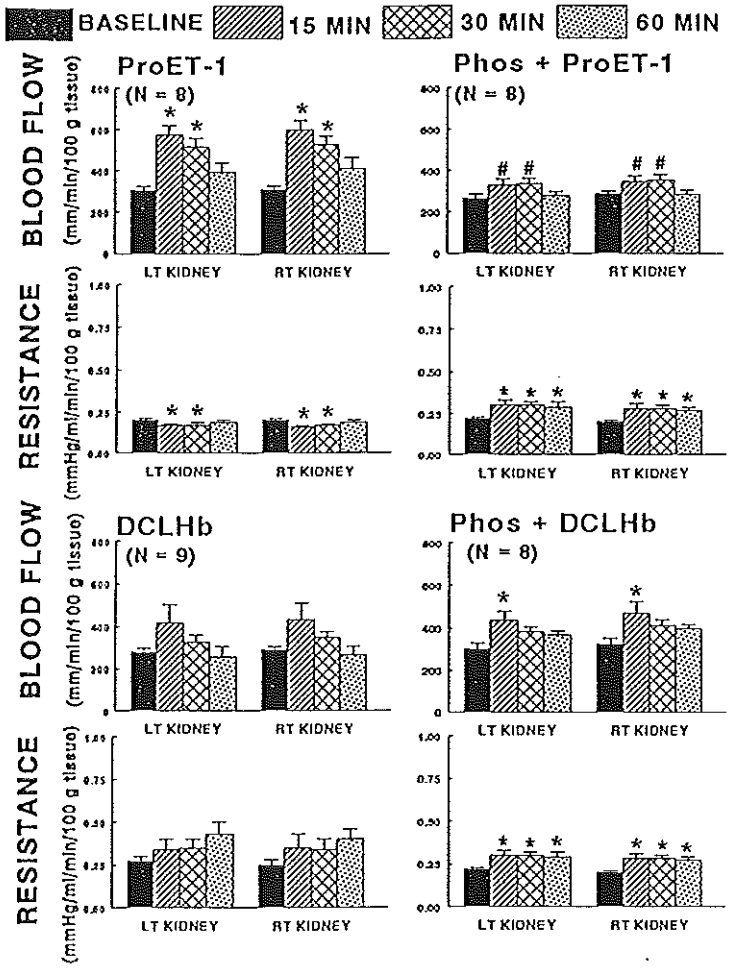

Fig. 3 The effect of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the kidneys before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated) and phosphoramidon (4 mg/kg, i.v.; 30 sec before the administration of proET-1 or DCLHb) treated rats. * Indicates significant difference as compared to baseline and # indicates significant difference as compared to control group.

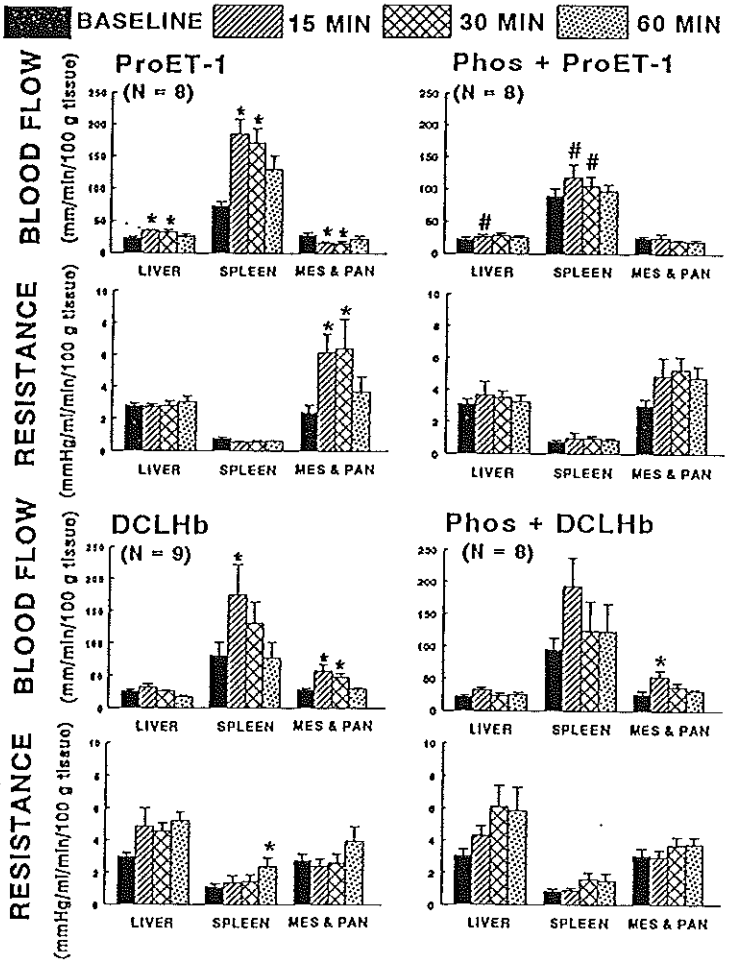

Fig. 4 The effect of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the liver, spleen and mesentery and pancreas (MES & PAN) before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated) and phosphoramidon (4 mg/kg, i.v.; 30 sec before the administration of proET-1 or DCLHb) treated rats. * Indicates significant difference as compared to baseline and # indicates significant difference as compared to control group.

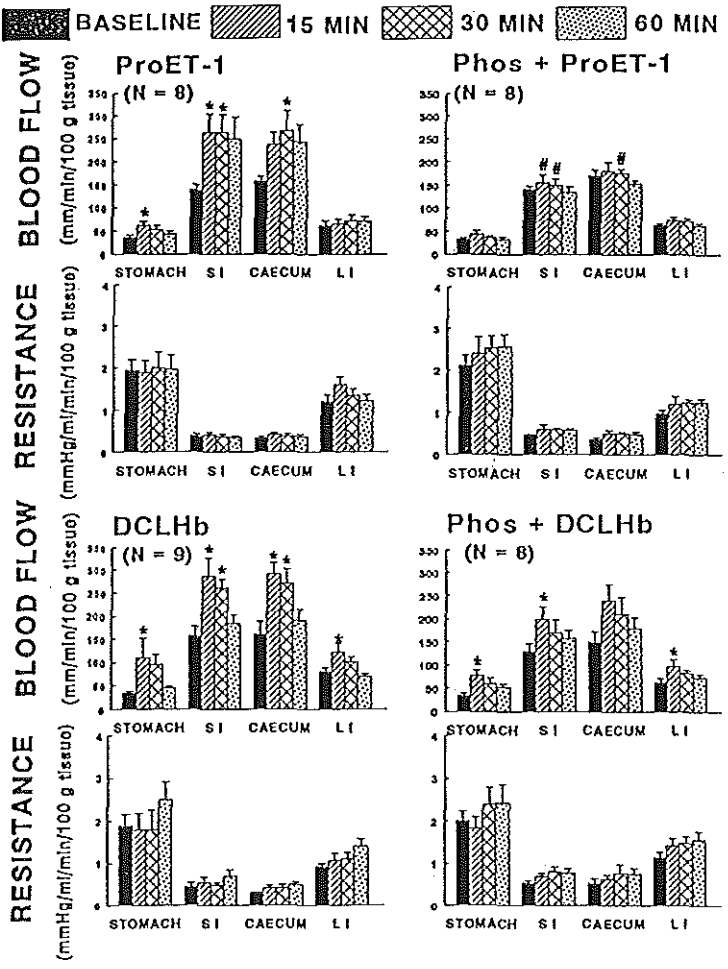

Fig. 5 The effect of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the stomach, small intestine (SI), caecum and large intestine (LI) before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated) and phosphoramidon (4 mg/kg, i.v.; 30 sec before the administration of proET-1 or DCLHb) treated rats. * Indicates significant difference as compared to baseline and # indicates significant difference as compared to control group.

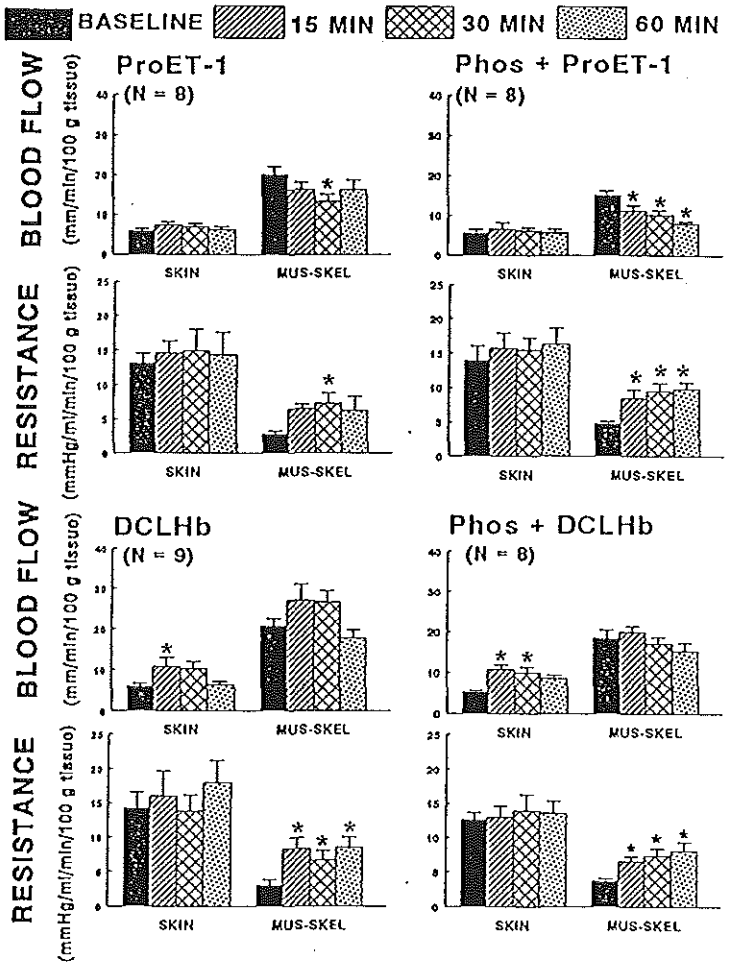

Fig. 6 The effect of proET-1 (20 μg/kg, i.v.) and DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the skin and musculo-skeletal system (MUS-SKEL) before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated) and phosphoramidon (4 mg/kg, i.v.; 30 sec before the administration of proET-1 or DCLHb) treated rats. * Indicates significant difference as compared to baseline and # indicates significant difference as compared to control group.

Effect of phosphoramidon on proET-1 induced changes in regional blood circulation

Administration of proET-1 (20 μg/kg, i.v.) produced a significant increase in blood flow to the cerebral hemispheres [F(3,28 = 3.59; p = 0.01], diencephalon [F(3,28) = 2.88; p = 0.01], cerebellum [F(3,28) = 2.71; p = 0.03] and brain stem [F(3,28) = 2.35; p = 0.03], and to the heart [F(3,28) = 8.42; p = 0.0003] of control (untreated) rats (Figures 1 and 2). ProET-1 increased blood flow to the left [F(3,28) = 8.55; p = 0.0004] and right kidneys [F(3,28) = 8.44; p = 0.0004] 15 and 30 min after administration to control rats (Figure 3). Administration of proET-1 also increased blood flow to the liver [F(3,28) = 3.91; p = 0.01] and spleen [F(3,28) = 6.4; p = 0.001] in control rats (Figure 4). ProET-1 increased blood flow to the stomach [F(3,28) = 2.01; p = 0.04], small intestine [F=(3,28) 2.51; p=0.03] and caecum [F=2.15; p = 0.03] after administration to control rats (Figure 5). ProET-1 significantly decreased blood flow to the musculo-skeletal system [F(3,28) = 1.86; p = 0.03] and mesentery and pancreas [F(3,28) = 2.46; p = 0.03] following administration to control rats (Figures 4 and 6).

ProET-1 produced a significant increase in vascular resistance in the musculo-skeletal system and mesentery and pancreas [F(3,28) = 2.57; p=0.03] after administration to control rats (Figures 4 and 6). A significant decrease in vascular resistance in the left [F(3,28) = 2.09; p = 0.05] and right kidneys [F(3,28) = 3.33; p = 0.01] was also observed following administration of proET-1 to control rats (Figure 3).

Phosphoramidon (4 mg/kg, i.v.) *per se* did not produce any significant change in regional blood circulation in rats (data not shown). Pretreatment with phosphoramidon (4 mg/kg, i.v.) significantly blocked the proET-1-induced increase in blood flow to the cerebral hemispheres [F(1,14) = 6.07; p = 0.03], diencephalon [F(1,14) = 6.26; p = 0.02], and cerebellum [F(1,14) = 5.87; p = 0.03] (Figure 1). Phosphoramidon significantly attenuated the proET-1-induced increase in blood flow to the heart [F(1,14) = 5.61; p = 0.03], and left [F(1,14) = 18.39; p = 0.0008] and right kidneys [F(1,14) = 21.63; p = 0.0004] (Figures 2 and 3). Pretreatment with phosphoramidon significantly attenuated proET-1-induced increase in blood flow to the small intestine [F(1,14) = 7.27; p = 0.02] and caecum [F(1,14) = 4.25; p = 0.05] (Figure 5). Phosphoramidon pretreatment significantly blocked the proET-1-induced increase in blood flow to the liver [F(1,14) = 5.58; p = 0.03] and spleen [F(1,14) = 4.56; p = 0.05] (Figure 4). Phosphoramidon pretreatment did not block the decrease in blood flow induced by proET-1 to the musculo-skeletal system (Figure 6).

The increase in vascular resistance in the musculo-skeletal system [F(3,28) = 5.03; p = 0.004] induced by proET-1 was not blocked by phosphoramidon pretreatment (Figure 6). However, phosphoramidon pretreatment significantly attenuated the proET-1-induced decrease in vascular resistance in the left [F(1,14) = 8.1; p = 0.003] and right kidneys [F(1,14) = 12.8; p = 0.003] (Figure 3). The increase in vascular resistance in mesentery and pancreas was not observed in phosphoramidon pretreated rats (Figure 4).

Effect of phosphoramidon on DCLHb induced changes in systemic hemodynamics

DCLHb (400 mg/kg, i.v.) produced a significant [$F(3,32) = 2.34$; $p = 0.05$] increase in MAP (63%) and TPR (54%) following administration in control (untreated) rats. The HR was not significantly affected following administration of DCLHb (Table 2).

In phosphoramidon (4 mg/kg, i.v.) pretreated rats DCLHb produced a significant increase in HR (25%), MAP (99%), CO (37%), SV (16%) and TPR (60%). Phosphoramidon pretreatment did not block any of the systemic hemodynamic effect produced by DCLHb (Table 2). The effect of DCLHb on MAP was accentuated which was accompanied by an increase in HR in phosphoramidon pretreated rats.

Effect of phosphoramidon on DCLHb induced changes in regional blood circulation

Administration of DCLHb (400 mg/kg, i.v.) significantly increased blood flow to the heart [$F(3,32) = 2.24$; $p = 0.04$] in control rats (Figure 2). The blood flow was also significantly increased to the stomach [$F(3,32) = 2.24$; $p = 0.04$], small intestine [$F(3,32) = 5.29$; $p = 0.003$], caecum [$F(3,32) = 5.24$; $p = 0.004$], and large intestine [$F(3,32) = 3.39$; $p = 0.04$] following administration of DCLHb (Figure 5). DCLHb increased blood flow to the spleen [$F(3,32) = 2.20$; $p = 0.05$], mesentery and pancreas [$F(3,32) = 4.74$; $p = 0.007$], and skin [F(3.32) 2.71; $p = 0.04$] following administration in control rats (Figures 4 and 6). The blood flow to the other regions was not significantly affected following administration of DCLHb.

DCLHb significantly increased vascular resistance in the musculo-skeletal system [$F(3,32) = 5.10$; $p = 0.005$] and spleen [$F(3,28) = 1.63$; $p = 0.05$] when administered to control rats (Figures 4 and 6). The vascular resistance in other regions was not significantly affected following administration of DCLHb to control rats.

In phosphoramidon (4 mg/kg, i.v.) pretreated rats, DCLHb produced a significant increase in blood flow to the cerebral hemispheres [$F(3,28) = 2.42$; $p = 0.03$], cerebellum [$F(3,28) = 2.78$; $p = 0.03$] and brain stem [$F(3,28) = 2.22$; $p = 0.05$] (Figure 1). DCLHb also produced a significant increase in blood flow to the left [$F(3,28) = 3.45$; $p = 0.006$] and right kidneys [$F(3,28) = 3.05$; $p = 0.009$] following administration to phosphoramidon treated rats (Figure 3). Phosphoramidon pretreatment did not block any of the blood flow changes induced by DCLHb.

DCLHb significantly increased the vascular resistance in the musculo-skeletal system [$F(3,28) = 4.4$; $p = 0.004$] when administered to phosphoramidon treated rats (Figure 6). Phosphoramidon pretreatment did not block any of the vascular resistance changes induced by DCLHb. An increase in vascular resistance was observed in the kidneys following administration of DCLHb to phosphoramidon treated rats (Figure 3).

10.4 Discussion

DCLHb was found to produce a significant pressor effect which was due to a significant increase in TPR and a small increase in SV and CO. The blood flow to the heart, GIT, portal system and skin was found to be increased following administration of DCLHb. Vascular resistance was increased significantly in the musculo-skeletal system. These cardiovascular effects of DCLHb were similar to those observed earlier (Sharma and Gulati, 1994; Sharma *et al.*, 1994). The present studies were conducted to determine whether the cardiovascular effects of DCLHb are due to an increase in the conversion of proET-1 to ET-1 as a result of stimulation of ECE. Phosphoramidon has been shown to block the conversion of proET-1 to ET-1 by inhibiting the activity of ECE (Matsumura *et al.*, 1990a; Matsumura *et al.*, 1990b). Phosphoramidon has no cardiovascular effects by itself but produces significant attenuation of most of the cardiovascular responses to human proET-1[1-38] in conscious Long Evans rats (Gardiner *et al.*, 1991). Present studies conducted in urethane anesthetized rats clearly demonstrate that the cardiovascular effects of proET-1 could be significantly blocked by pretreatment with phosphoramidon. However, DCLHb induced cardiovascular effects could not be attenuated by pretreatment with phosphoramidon.

ProET-1 produced a significant increase in blood pressure in rats similar to that observed by others (Haleen *et al.*, 1993; Hoffman *et al.*, 1994). The increase in blood pressure was due to a significant increase in TPR, while CO was not affected by the administration of proET-1. ProET-1 was found to produce a decrease in blood flow and increase in vascular resistance in the musculo-skeletal system and mesentery and pancreas. Vascular resistance was not affected in the brain, heart, liver, spleen, GIT and skin but was decreased in the kidneys. An increase in blood flow was observed in the brain, heart, kidneys, liver, spleen and GIT. Phosphoramidon did not block the proET-1 induced increase in vascular resistance and decrease in blood flow to the musculo-skeletal system, but the increase in vascular resistance and decrease in blood flow in the mesentery and pancreas was attenuated by phosphoramidon pretreatment. Phosphoramidon also attenuated the increase in blood flow induced by proET-1 in the brain, heart, kidneys, liver, spleen and GIT. Thus most of the cardiovascular effects of proET-1 were significantly attenuated by phosphoramidon. These results are similar to several other studies indicating that phosphoramidon attenuated the cardiovascular effects of proET-1 (Le Monnier de Gouville and Cavero, 1991). It has also been found that intra-arterial injections of proET-1 produced a slowly developing increase in hindquarters perfusion pressure in cats which could be blocked by phosphoramidon (Tamura *et al.*, 1994). Phosphoramidon attenuated a dose-dependent decrease in skin blood flow induced by local administration of proET-1 in rats (Lawrence and Brain, 1993). However, it has also been found that the degree of inhibition of the

regional hemodynamic effects of proET-1 by phosphoramidon differs in different vascular beds (Gardiner *et al.*, 1991).

It has been found that DCLHb produces an increase in plasma ET-1 like immunoreactivity, (Gulati *et al.*, 1995) and it could be possible that some of the cardiovascular effects of DCLHb could be due to an increase in circulating ET-1 levels. Further studies indicate that the cardiovascular effects of DCLHb could be significantly attenuated by pretreatment with BQ-123, a specific ET_A receptor antagonist (Gulati *et al.*, 1996). These studies suggest that cardiovascular effects of DCLHb may be mediated through the ET system. However, in the present study phosphoramidon did not block any of the cardiovascular effects of DCLHb. These results do not agree with a previous study in which it was found that the pressor effect of DCLHb was substantially attenuated by phosphoramidon (5 mg/kg, i.v.) pretreatment (Schultz *et al.*, 1993). The differences could be attributed to the fact that (1) a lower dose (280 mg/kg, i.v.) of DCLHb was used in the previous study while we used a higher dose (400 mg/kg, i.v.) of DCLHb; (2) Urethane anesthetized rats were used in the present study while Schultz et al. used conscious rats (Schultz *et al.*, 1993); (3) The baseline MAP was lower in the present study as compared to the other study. The rats used by Schultz et al. showed significantly different baseline values of MAP in the DCLHb and phosphoramidon + DCLHb groups while the baseline values in our group are similar in both the groups (Schultz *et al.*, 1993). We observed a significantly greater pressor effect with DCLHb in phosphoramidon treated rats. The accentuation in the pressor effect of DCLHb appears to be due to an increase in cardiac output, which was the result of a significant increase in HR but not in SV in phosphoramidon treated rats. It is interesting to note that blood flow to the brain and kidneys by DCLHb was increased in phosphoramidon treated rats. This could be attributed to the decrease in the conversion of endogenous proET-1 to ET-1 by ECE and thus reducing the vascular tone in these vascular beds. Differences in the action of phosphoramidon in different vascular beds has been shown earlier (Gardiner *et al.*, 1991).

It has been reported that phosphoramidon inhibits the conversion of exogenously applied proET-1 to ET-1 more effectively than endogenous production of ET-1 in cultured vascular smooth muscle cells (Ikegawa *et al.*, 1991). Much higher concentration of phosphoramidon were required to inhibit the conversion of endogenous proET-1 in ECE-1 cDNA transfected intact cells as compared with the concentrations required for the *in vitro* inhibition (Xu *et al.*, 1994). In another study, a marked difference in the inhibitory potencies of phosphoramidon between the release of endogenous ET-1 and ET-1 generation from exogenously applied proET-1 was observed (Matsumura *et al.*, 1995) Phosphoramidon suppressed ET-1 generation from exogenously applied proET-1 by 90-95% at 5×10^{-5}M concentration, whereas the release of endogenous ET-1 was suppressed by only 45% at a higher concentration of 2×10^{-4}M (Matsumura *et al.*, 1995). It could be possible that in the present study the conversion of proET-1 to ET-1 of exogenously administered proET-1 is effectively inhibited as compared to an increase

in the endogenous conversion of proET-1 to ET-1 due to stimulation of ECE by DCLHb. We performed two experiments using double the dose of phosphoramidon (8 mg/kg, i.v.), and found that at even at this higher dose, phosphoramidon did not attenuate the cardiovascular effects of DCLHb (data not shown).

It is concluded from the present study that proET-1 produces significant cardiovascular effects which could be blocked by pretreatment with ECE inhibitor phosphoramidon. DCLHb, which has been shown to increase circulating ET-1 like immunoreactivity, also produced significant cardiovascular effects which could not be blocked by phosphoramidon. It appears that the cardiovascular effects of DCLHb are not due to enhancement of ET-1 production including stimulation of ECE in anesthetized rats.

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Chapter 11

Role of endothelin mechanism in the cardiovascular effects of diaspirin crosslinked and stroma reduced haemoglobin

Summary

Diaspirin crosslinked haemoglobin (DCLHb™; Baxter Healthcare Corp., Round Lake, IL, USA) is a resuscitative solution with excellent oxygen carrying capacity. DCLHb has been found to produce an immediate increase in blood pressure and marked regional circulatory changes in rats and pigs. The present study was conducted to determine the role of endothelin mechanisms in the cardiovascular actions of DCLHb (modified) and (unmodified) stroma reduced haemoglobin solutions (SRHb). Infusion of SRHb (400 mg/kg, i.v.) in control rats produced an increase in blood pressure (43%) and total peripheral resistance (65%) without any change in heart rate, cardiac output and stroke volume. SRHb decreased blood flow to the kidneys and liver, increased blood flow to the heart and had no effect on blood flow to the brain, gastrointestinal tract, spleen, musculo-skeletal system, skin, mesentery and pancreas. Infusion of SRHb in rats treated with BQ-123 (5 mg/kg/h, i.v.) increased the blood pressure similar to control rats, but the increase in total peripheral resistance was significantly attenuated. The SRHb induced decrease in blood flow to the kidneys and liver was significantly attenuated in BQ-123 treated rats as compared to control rats. However, the SRHb induced increase in blood flow to the heart of BQ-123 treated rats was similar to control rats. Infusion of DCLHb (400 mg/kg, i.v.) produced an increase in blood pressure (81%), cardiac output (36%), stroke volume (30%) and total peripheral resistance (45%) along with an increase in blood flow to the heart, spleen, gastrointestinal tract and skin of control rats. The blood flow to the brain, kidney, liver, musculoskeletal system, mesentery and pancreas was not altered by DCLHb in control rats. The increase in blood pressure, cardiac output, stroke volume and total peripheral resistance by DCLHb was significantly blocked in BQ-123 treated rats as compared to control rats. The increase in blood flow to the heart, spleen and skin by DCLHb was significantly blocked in BQ-123 treated rats as compared to control rats. DCLHb produced an increase in the blood flow to the brain and a decrease in blood flow to the kidney and musculoskeletal system of BQ-123 treated rats as compared to control rats. Blood plasma endothelin-1 like immunoreactivity was found to be significantly increased following treatment with DCLHb or SRHb. It can be concluded that the endothelin-A receptor antagonist, BQ-123, could attenuate the systemic hemodynamic and regional circulatory effects of DCLHb and SRHb. However, the increase in blood flow to the heart induced by SRHb could not be attenuated by BQ-123.

11.1 Introduction

DCLHb, a blood substitute, derived from the haemoglobin of outdated erythrocytes has been developed by cross-linking molecular haemoglobin between the α-subunits by reaction with the diaspirin compound, bis (3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The purification process associated with the manufacture of DCLHb includes heat pasteurization of the solution (Estep *et al.*, 1989b; Estep *et al.*, 1989a). DCLHb has been found to be biochemically stable and possesses excellent oxygen carrying capacity (Chatterjee *et al.*, 1986). The cross-linking of the α subunits affords the haemoglobin a favorable oxygen dissociation curve (Snyder *et al.*, 1987; Vandegriff *et al.*, 1989).

It has been demonstrated in swine that after partial or complete exchange transfusion with DCLHb, cardiac and renal functions are not affected significantly (Hess *et al.*, 1989). DCLHb was also found to be a promising resuscitative fluid after hemorrhage (Przybelski *et al.*, 1990). Resuscitation with DCLHb (10 ml/kg of 14%) was as efficacious as nearly twice the volume of whole blood in the restoration of cardiovascular and tissue oxygenation parameters (Przybelski *et al.*, 1991). DCLHb has been found to decrease the extent of focal cerebral ischemia induced by 10 min of middle cerebral artery occlusion in rats (Cole *et al.*, 1992). DCLHb increases the mean arterial pressure when administered to hemorrhagic rats (Przybelski *et al.*, 1991). DCLHb (125-4000 mg/kg), when administered intravenously, produced a 25-35 % increase in mean arterial pressure of conscious rats. The pressor effect was self-limiting and was not due to volume load or oncotic pressure (Hamilton *et al.*, 1992). Besides increasing blood pressure, DCLHb has been found to increase the blood flow to several organs (Sharma and Gulati, 1994). The mechanism(s) responsible for the regional circulatory actions of DCLHb have not been completely elucidated.

Endothelin, a 21 amino-acid peptide isolated from vascular endothelial cells, is one of the most potent regulators of vascular tone (Yanagisawa *et al.*, 1988). Several studies have demonstrated a widespread distribution of specific endothelin receptors (Gulati and Srimal, 1992). At present, two receptors have been identified, endothelin-A and endothelin-B. The endothelin-A receptor is highly selective for endothelin-1 and is characterized by the rank order of binding affinities: endothelin-1 > endothelin-2 > endothelin-3 (Arai *et al.*, 1990). The endothelin-B receptor is non-selective (endothelin-1 = endothelin-2 = endothelin-3) (Sakurai *et al.*, 1990). BQ-123 (cyclo(D-Asp-L-Pro-D-Val-L-Leu-D-Trp)) antagonizes endothelin-1 induced contraction of porcine isolated coronary artery strips and has greater affinity for endothelin-A receptors in porcine aortic smooth muscle than for endothelin-B receptors in porcine cerebellum (Ihara *et al.*, 1992). Endothelin-1 is believed to primarily act as regulator of regional blood flow as a vasoconstrictor of peripheral vessels modulating and balancing the vasodilator action of nitric oxide and prostacyclin. All of these vasoactive compounds are produced by endothelial

cells (Vane and Botting, 1993). However, the plasma concentration of endothelin-1 is too low to maintain vascular tone (Fukuda *et al.*, 1988). The endothelin-A receptor antagonist, BQ-123, when injected intravenously into rats, did not affect the basal blood pressure (Ihara *et al.*, 1992), reinforcing the conclusion that circulating endothelin-1 is not a major factor in the maintenance of vascular tone. However, several factors have been suggested to stimulate the production of endothelin-1 in endothelial cells, resulting in the release of this agonist on the basal side of the endothelium and action upon the underlying smooth muscles to produce vasoconstriction (Wagner *et al.*, 1992). Endothelin-1 might therefore be participating in the maintenance of blood flow of peripheral small resistance vessels in a paracrine manner (Masaki, 1994). Interaction of DCLHb with both nitric oxide and endothelin have been implicated in mediating the pressor response in rats (Schultz *et al.*, 1993).

In the present study we have investigated the effect of BQ-123, a potent endothelin-A receptor antagonist, on the regional circulatory and systemic hemodynamic changes induced by an unmodified (SRHb) and by a modified and chemically stabilized haemoglobin solution (DCLHb). The effect of stroma reduced and DCLHb solutions on blood plasma endothelin-1 like immunoreactivity was also determined.

11.2 Materials and Methods

Drugs and Chemicals

Diaspirin cross-linked haemoglobin was prepared and provided by Baxter Healthcare Corp., Round Lake, IL, USA. SRHb was prepared at Baxter Healthcare Corp., Round Lake, IL, USA by a method similar to that described earlier (Zager and Gamelin, 1989). Briefly, outdated human RBCs were separated by centrifugation and lysed by adding 2 vol of distilled water per 1 vol of RBCs followed by two freeze thawing procedures. The lysed cells were centrifuged for 15 min at 26,000 g. The supernatant containing SRHb was collected and used for subsequent testing. The physicochemical properties of SRHb and DCLHb are shown in Table 1. BQ-123 was purchased from American Peptide Co. Inc., Sunnyvale, CA, USA. [^{125}I]Tyr^{13}-endothelin-1 (human, porcine) was purchased from DuPont NEN Research Products, Wilmington, DE, USA. SEP-Columns (200 mg), rabbit anti-endothelin-1 (porcine, human) serum, normal rabbit serum and goat anti-rabbit IgG serum were purchased from Peninsula Laboratories Inc., Belmont, CA, USA. Radioimmunoassay buffer consisted of 19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 0.05 M NaCl, 0.1 % bovine serum albumin, 0.1 % Triton X-100 and 0.01 % NaN_3.

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI, USA) weighing 340-390 g were used in the study according to a protocol approved by the University Animal Care Committee. Systemic hemodynamics and regional blood circulation were determined using a

radioactive microsphere technique (Sharma and Gulati, 1994). Rats were anesthetized with urethane (1.5 g/kg, intraperitoneally). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a P23 ID pressure transducer (Gould Inc., Tst and Mgmt. Rec. Syst. Div., Valley View, OH, USA) for recording the blood pressure on a P7D polygraph through a 7PI preamplifier (Grass Instrument Co., Quincy, MA, USA). The heart rate was recorded through a 7P4B Grass tachograph, triggered from the blood pressure signals. In order to keep the blood pO_2, pCO_2 and pH constant and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a rodent ventilator (Model 683; Harvard Apparatus, Inc. S. Natick, MA, USA). The carotid artery of the right side was exposed and a PE 50 cannula was guided through the common carotid artery to the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P23 DC pressure transducer. When the cannula reached the ventricle the diastolic pressure dropped to zero. The femoral artery of the right side was cannulated and connected to a withdrawal pump (Model 22; Harvard Apparatus, Inc. S. Natick, MA, USA).

Determination of systemic hemodynamics and regional circulation

At each measurement, a suspension of approximately 200,000 microspheres (15 $\pm$ 1 μm diameter) labeled with either ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA, USA) in 0.2 ml saline were injected into the left ventricle after thorough mixing. The line was then flushed with 0.4 ml saline over a 15 sec period to ensure delivery of the total dose of microspheres. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the right femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before each microsphere injection. At the end of the experiment the animals were sacrificed with an overdose of pentobarbital sodium and all the tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: lungs, heart, liver, stomach, small intestine, caecum, large intestine, mesentery and pancreas, spleen, left kidney, right kidney, cerebral hemispheres, diencephalon, cerebellum, brain stem, skin and the rest of the body consisting of muscles and bones. The radioactivity in the standards, the blood samples and the tissue samples were counted in a gamma counter (Minaxi Auto-Gamma 5000 series; Packard Instrument Co. Downers Grove, IL, USA) with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output, (2) stroke volume, (3) total peripheral resistance, (4) regional blood flow [(withdrawal rate of arterial blood sample x radioactivity in tissues)/radioactivity in sampled arterial blood] and (5) regional vascular resistance (mean arterial pressure/regional blood flow). Infusion of saline did not affect the systemic hemodynamics and regional blood circulation. Five groups of animals were used: (1) Saline (4 ml/kg) was administered

intravenously in normal untreated rats (no effect on cardiovascular parameters, data not presented), (2) SRHb (400 mg/kg) was administered intravenously in normal untreated rats, (3) SRHb (400 mg/kg) was administered intravenously in BQ-123 (5 mg/kg/h, i.v. infusion for 75 min) treated rats, (4) DCLHb (400 mg/kg) was administered intravenously in normal untreated rats and (5) DCLHb (400 mg/kg) was administered intravenously in BQ-123 (5 mg/kg/h, i.v. infusion for 75 min) treated rats. In treated rats haemoglobin solutions were administered 15 min after starting the infusion of BQ-123. The infusion of BQ-123 was continued until the end of each experiment. The doses were selected on the basis of studies conducted previously (Hamilton *et al.*, 1992; Sharma and Gulati, 1994) which demonstrated that the infusion of this dose of DCLHb resulted in a near maximal pressor response. The dose of BQ-123 was selected on the basis of results obtained in our laboratory in which BQ-123 (5 mg/kg/h, i.v.) produced very significant blockade of endothelin-1 induced systemic hemodynamics and regional circulatory effects in rats (Sharma and Gulati, 1995). Similar results with BQ-123 have been obtained in other laboratories (Douglas *et al.*, 1992; Wong *et al.*, 1994).

Determination of endothelin-1 concentration by radioimmunoassay

The animals were sacrificed at 15, 30 and 60 min after administration of saline, DCLHb or SRHb. Blood samples from the femoral vein were collected in plastic tubes containing ethylenediaminetetraacetate (1 mg/ml) and aprotinin (500 kIU/ml). Samples were centrifuged (15 min, 4 °C, 1000 x g) and the plasma was collected. Plasma was mixed with an equal volume of 20% acetic acid and centrifuged (15 min, 4 °C, 1000 x g) and the supernatant was collected for subsequent assay. All reagents were kept ice cold and all procedures were performed in the cold. The SEP-columns were activated by successively washing with 3 ml methanol, 2 ml water and 2 ml of 10% acetic acid solution. The samples collected as described earlier were slowly applied on the column and then allowed to flow through the column at the rate determined by gravity. The columns were washed with 2 ml of 10% acetic acid followed by 3 ml of ethyl acetate. Endothelin was eluted with 1.5 ml of elution buffer (1 : 4; 0.05 M ammonium bicarbonate solution : methanol) into polypropylene tubes. The eluate was evaporated to dryness under a gentle stream of nitrogen gas. The recovery was found to be 86% by this method. Radioimmunoassay was performed using antibodies specific for endothelin-1 (Peninsula Lab. Inc, Belmont, CA, USA). This antibody has only 7% cross reactivity with endothelin-2 or endothelin-3. The incubation mixture for radioimmunoassay consisted of 100 µl of standards or samples and 100 µl of rabbit anti-endothelin-1 serum. The tubes were vortexed and incubated overnight at 4 °C. The next day, [^{125}I] endothelin-1 (100 µl) was added and the mixture vortexed and incubated for 24 hours. On day 3, goat anti-rabbit IgG serum (100 µl) and normal rabbit serum (100 µl) were added to all the tubes, vortexed and incubated for 2 h at room temperature. Radioimmunoassay buffer (500 µl) was added and the tubes were vortexed and then centrifuged at 1,700 g for 20 min. The supernatant was carefully aspirated and the radioactivity in the pellet

was determined using a gamma counter (Model Cobra 5005; Packard Instrument Co. Downers Grove, IL, USA). The concentration of endothelin-1 was determined from standard plots of % B/B_0 versus log dose (pg) and expressed as pg/ml in plasma and pg/g wet weight in tissues. Three groups of normal rats received intravenous infusions of: (1) saline (4 ml/kg), (2) SRHb (400 mg/kg), and (3) DCLHb (400 mg/kg).

Statistics

All data are presented as mean values ± SEM. Mean blood pressure (BP; mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by analysis of variance followed by Duncan's test and by Student's t test. Each group consisted of 5 to 6 animals and the level of $P < 0.05$ was considered significant.

11.3 Results

Effect of DCLHb and SRHb on endothelin-1 concentrations in blood plasma

Haemoglobin solutions (DCLHb and SRHb) significantly ($P = 0.0001$) increased the concentration of endothelin-1 like immunoreactivity in blood plasma obtained 15, 30 and 60 min after administration in rats. The endogenous concentration of endothelin-1 in blood plasma of control rats without any treatment was found to be 6.07 ± 0.59 pg/ml. The concentration of endothelin-1 at 15 min of treatment was found to be 6.81 ± 0.67 pg/ml in vehicle treated rats, 17.63 ± 1.22 pg/ml in DCLHb and 24.01 ± 2.64 pg/ml in SRHb treated rats. At 30 min of treatment the concentration of endothelin-1 was found to be 4.67 ± 0.61 pg/ml, 12.49 ± 0.31 pg/ml and 9.70 ± 0.23 pg/ml in vehicle, DCLHb and SRHb treated rats, respectively. At 60 min of treatment the concentration of endothelin-1 was found to be 4.56 ± 0.44 pg/ml, 7.88 ± 0.48 pg/ml and 10.60 ± 1.77 pg/ml in vehicle, DCLHb and SRHb treated rats, respectively. Plasma endothelin-1 levels were maximum at 15 min and significantly decreased at 30 and 60 min after the administration of diaspirin crosslinked and SRHb solutions. The concentration of blood plasma endothelin-1 like immunoreactivity at 15 min was found to be significantly ($P < 0.05$) greater in SRHb treated rats as compared to DCLHb treated rats.

Effect of BQ-123 pretreatment on SRHb and DCLHb induced changes in systemic hemodynamics

Infusion of SRHb into control (untreated) rats produced a significant increase in blood pressure ($P = 0.04$) and total peripheral resistance ($P = 0.04$), while heart rate, cardiac output and stroke volume were not affected (Table 2). When SRHb was administered to BQ-123 treated rats, there was also a significant increase in blood pressure, but an increase in total peripheral resistance was not observed. The SRHb induced increase in total peripheral resistance was significantly ($P = 0.03$) blocked in BQ-123 treated rats as compared to control rats. SRHb

produced no change in heart rate, cardiac output and stroke volume in BQ-123 treated rats (Table 2). Infusion of DCLHb produced a significant increase in blood pressure ($P < 0.0001$) and total peripheral resistance ($P = 0.03$), while heart rate, cardiac output and stroke volume were not affected in control (untreated) rats (Table 3). DCLHb, when administered to BQ-123 treated rats, produced a slight increase in blood pressure, but an increase in total peripheral resistance was not observed. DCLHb produced no change in heart rate, cardiac output and stroke volume in BQ-123 treated rats. The pressor effect and increase in total peripheral resistance induced by DCLHb were significantly ($P = 0.001$) blocked in BQ-123 treated rats as compared to control rats (Table 3).

Effect of BQ-123 pretreatment on SRHb induced changes in regional circulation

Infusion of SRHb (400 mg/kg, i.v.) produced no change in blood flow to the brain, gastrointestinal tract, spleen, mesentery and pancreas, skin and musculoskeletal system of control (untreated) rats. SRHb produced a significant increase in blood flow to the heart ($P = 0.004$) and decrease to the kidneys ($P = 0.05$) and liver ($P = 0.004$) of control rats (Figures 1 and 2). However, the blood flow was found to be significantly increased in the gastrointestinal tract ($P = 0.008$), skin ($P = 0.0007$) and heart ($P = 0.003$) of BQ-123 pretreated rats. No change in blood flow was observed in the brain, kidneys, liver, spleen, mesentery and pancreas and musculoskeletal system of BQ-123 treated rats. BQ-123 pretreatment attenuated the decrease in blood flow to the liver and kidneys, did not affect the increase in blood flow to the heart, and induced an increase in blood flow to the skin ($P = 0.0007$) and gastrointestinal tract ($P = 0.04$) upon infusion of SRHb (Figures 1 and 2).

Vascular resistance was significantly increased in the kidneys ($P = 0.05$), liver ($P = 0.03$), spleen ($P = 0.03$), mesentery and pancreas ($P = 0.03$), musculoskeletal system ($P = 0.03$) and skin ($P = 0.04$) of control rats. There was no change in vascular resistance in the brain, heart and gastrointestinal tract following infusion of SRHb in control rats. However, the vascular resistance was significantly ($P = 0.002$) decreased in the heart of BQ-123 treated rats as compared to control rats following infusion of SRHb (Figures 1 and 2). BQ-123 pretreatment attenuated the increase in vascular resistance induced by SRHb in most of the organs but its effect in the kidneys, skin and heart was completely blocked (Figures 1 and 2).

TABLE 1. Physicochemical properties of unmodified (SRHb) and modified (DCLHb) haemoglobin solutions.

	SRHb	DCLHb
Total haemoglobin (g/dl)	10.4 g/dl	10.0 g/dl
Pyrogenicity (LAL) EU/ml	< 0.06	< 0.125
Methaemoglobin %	12.8 %	4.1 %
Sodium (mEq/l)	114	141
Chloride (mEq/l)	137	112
Sterility	Sterile	Sterile
Phospholipids	>50 ppm	0.2 ppm
Cross-linking	-------	99.9 %
p50	-------	31 mmHg

TABLE 2. Effect of SRHb (SRHb; 400 mg/kg, i.v.) on the systemic hemodynamics of control (untreated; N = 5) and BQ-123 (5 mg/kg/h, i.v.; N = 6) treated rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)				
SRHb	320 ± 30	374 ± 16	374 ± 15	336 ± 34
BQ-123+ SRHb	368 ± 10	383 ± 13	385 ± 13	375 ± 21
Blood pressure (mmHg)				
SRHb	85.0 ± 5.3	121.2 ± 8.9*	116.9 ± 10*	102.3 ± 10.6
BQ-123+ SRHb	78.2 ± 6.4	121.5 ± 5.6*	110.4 ± 6.3*	88.3 ± 7.6
Cardiac output (ml/min)				
SRHb	95.5 ± 8.9	129.6 ± 28.1	117.1 ± 20.9	69.3 ± 5.4
BQ-123+ SRHb	99.8 ± 9.1	123.6 ± 6.5	118.29 ± 9.52	89.4 ± 8.1
Stroke volume (ml)				
SRHb	0.32 ± 0.07	0.34 ± 0.08	0.32 ± 0.06	0.22 ± 0.04
BQ-123+ SRHb	0.27 ± 0.02	0.32 ± 0.01	0.39 ± 0.09	0.24 ± 0.02
Total peripheral resistance (mmHg/l/min)				
SRHb	920 ± 91	1058 ± 169	1119 ± 196	1514 ± 187*
BQ-123+ SRHb	806 ± 74	996 ± 73	879 ± 147	1016 ± 107+

*Indicates significant ($P < 0.05$) difference as compared to baseline. +Indicates significant ($P < 0.05$) difference as compared to control (SRHb) group. The baseline values in BQ-123 treated rats are prior to administration of SRHb (or 15 min following BQ-123 treatment).

TABLE 3. Effect of DCLHb (DCLHb; 400 mg/kg, i.v.) on the systemic hemodynamics of control (untreated; N = 6) and BQ-123 (5 mg/kg/h, i.v.; N = 5) treated rats.

Parameter	Baseline	15 min	30 min	60 min
Heart rate (beats/min)				
DCLHb	373 ± 14	399 ± 10	400 ± 12	393 ± 9
BQ-123+ DCLHb	360 ± 20	363 ± 13	369 ± 11	370 ± 11
Blood pressure (mmHg)				
DCLHb	86.5 ± 4.1	156.8 ± 4.3*	150.1 ± 7.6*	126.8 ± 8.3
BQ-123+ DCLHb	83.8 ± 3.7	100.3 ± 7.0*+	98.2 ± 9.7+	83.0 ± 4.6+
Cardiac output (ml/min)				
DCLHb	88.1 ± 6.9	108.2 ± 7.8	120.1 ± 19.5	89.9 ± 8.4
BQ-123+ DCLHb	95.6 ± 6.0	110.1 ± 6.2	103.8 ± 4.9	77.1 ± 7.7
Stroke volume (ml)				
DCLHb	0.23 ± 0.02	0.27 ± 0.02	0.30 ± 0.05	0.23 ± 0.02
BQ-123+ DCLHb	0.27 ± 0.01	0.31 ± 0.03	0.28 ± 0.02	0.21 ± 0.02
Total peripheral resistance (mmHg/l/min)				
DCLHb	947 ± 79	1489 ± 121*	1486 ± 201*	1462 ± 143*
BQ-123+ DCLHb	879 ± 22	925 ± 84+	948 ± 90+	1117 ± 125

*Indicates significant ($P < 0.05$) difference as compared to baseline. +Indicates significant ($P < 0.05$) difference as compared to control (DCLHb) group. The baseline values in BQ-123 treated rats are prior to administration of DCLHb (or 15 min following BQ-123 treatment).

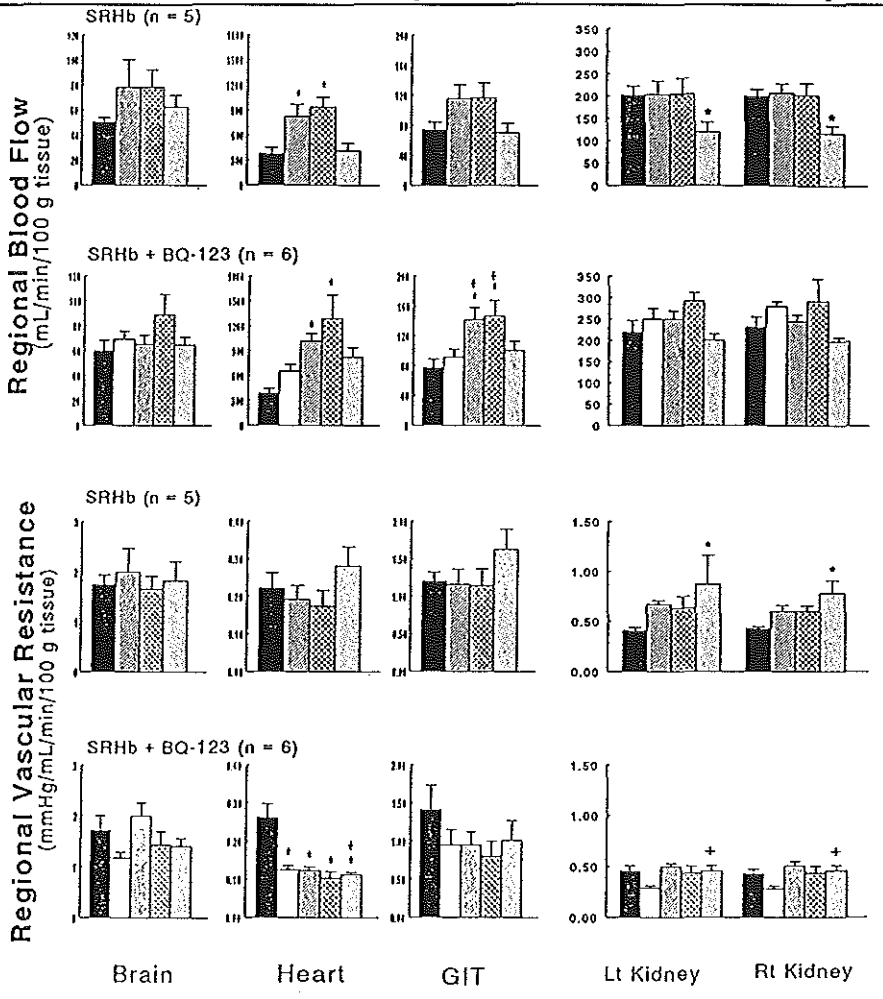

Fig. 1 The effect of SRHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the brain, heart, gastrointestinal tract (GIT), left and right kidneys before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated; N = 6) and BQ-123 (5 mg/kg/h, i.v. infusion starting 15 min before the administration of SRHb (SRHb); N = 5; hollow bars) treated rats. *Indicates significant difference as compared to baseline and $^{+}$indicates significant difference as compared to control (SRHb) group.

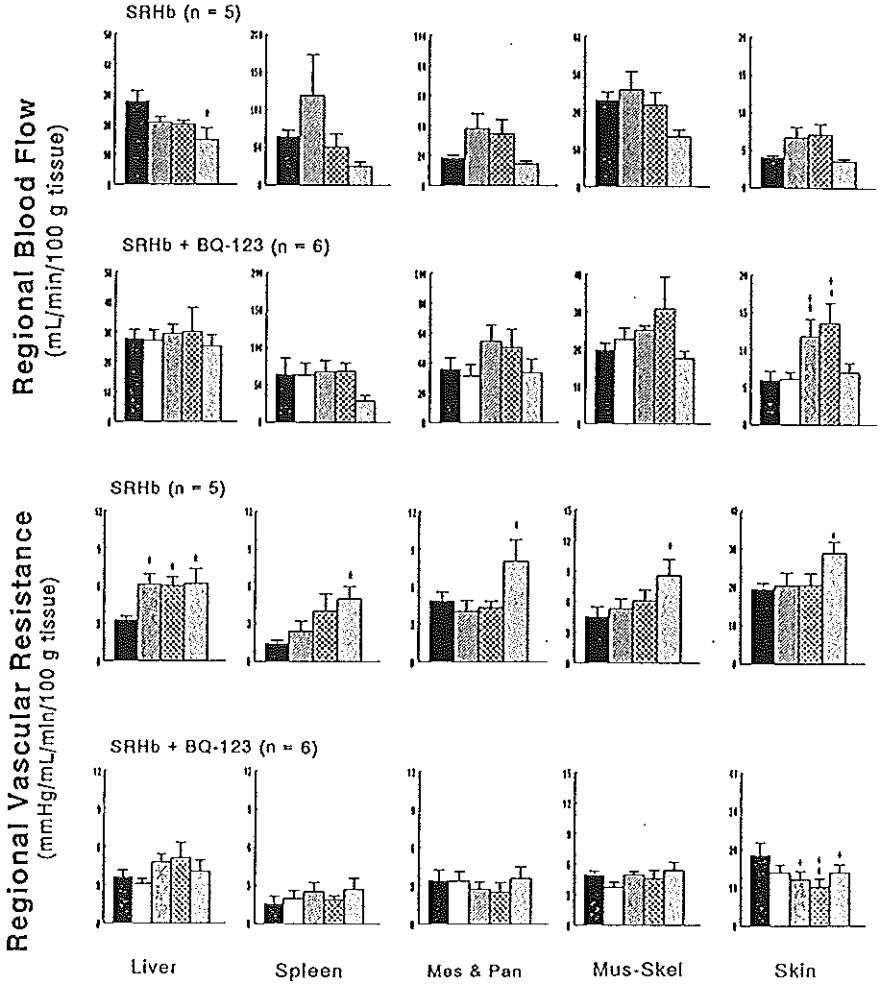

Fig. 2 The effect of SRHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the liver, spleen, mesentery and pancreas (Mes & Pan) musculoskeletal system (Mus-Skel) and skin before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated; N = 6) and BQ-123 (5 mg/kg/h, i.v. infusion starting 15 min before the administration of SRHb (SRHb); N = 5; hollow bars) treated rats. *Indicates significant difference as compared to baseline and $^{+}$indicates significant difference as compared to control (SRHb) group.

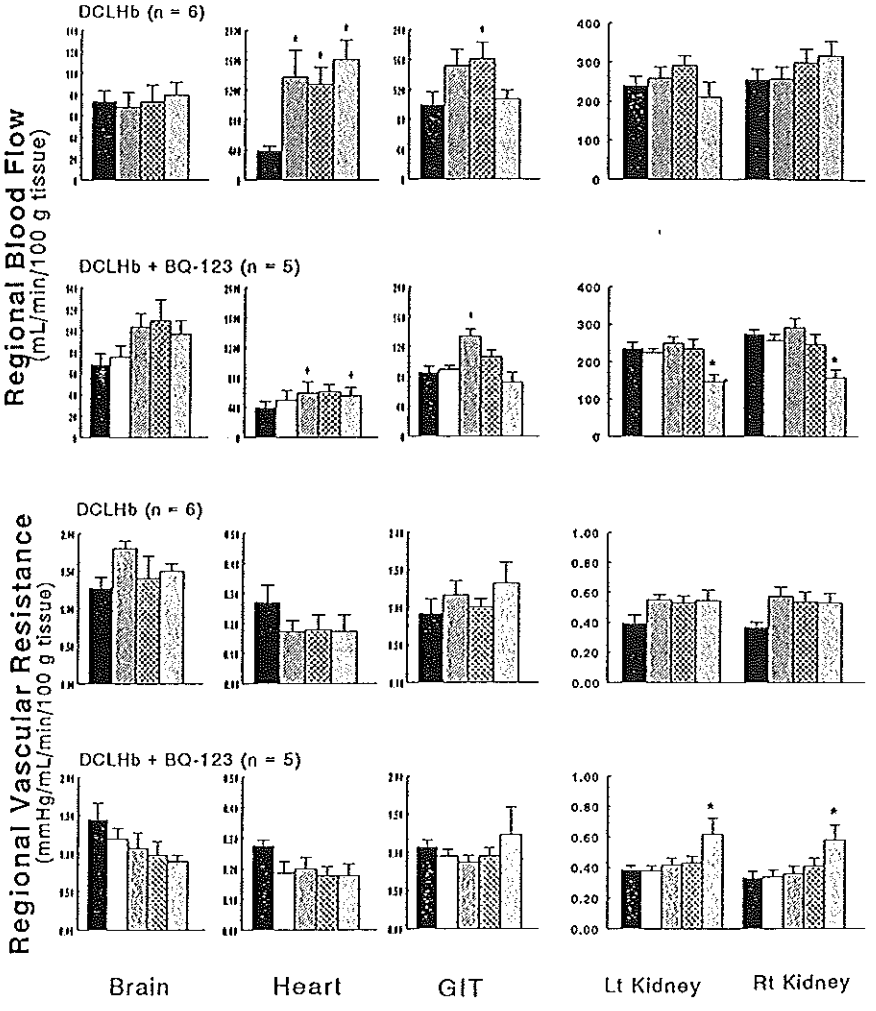

Fig. 3 The effect of DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the brain, heart, gastrointestinal tract (GIT), left and right kidneys before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated; N = 6) and BQ-123 (5 mg/kg/h, i.v. infusion starting 15 min before the administration of DCLHb (DCLHb); N = 5; hollow bars) treated rats. *Indicates significant difference as compared to baseline and ˙indicates significant difference as compared to control (DCLHb) group.

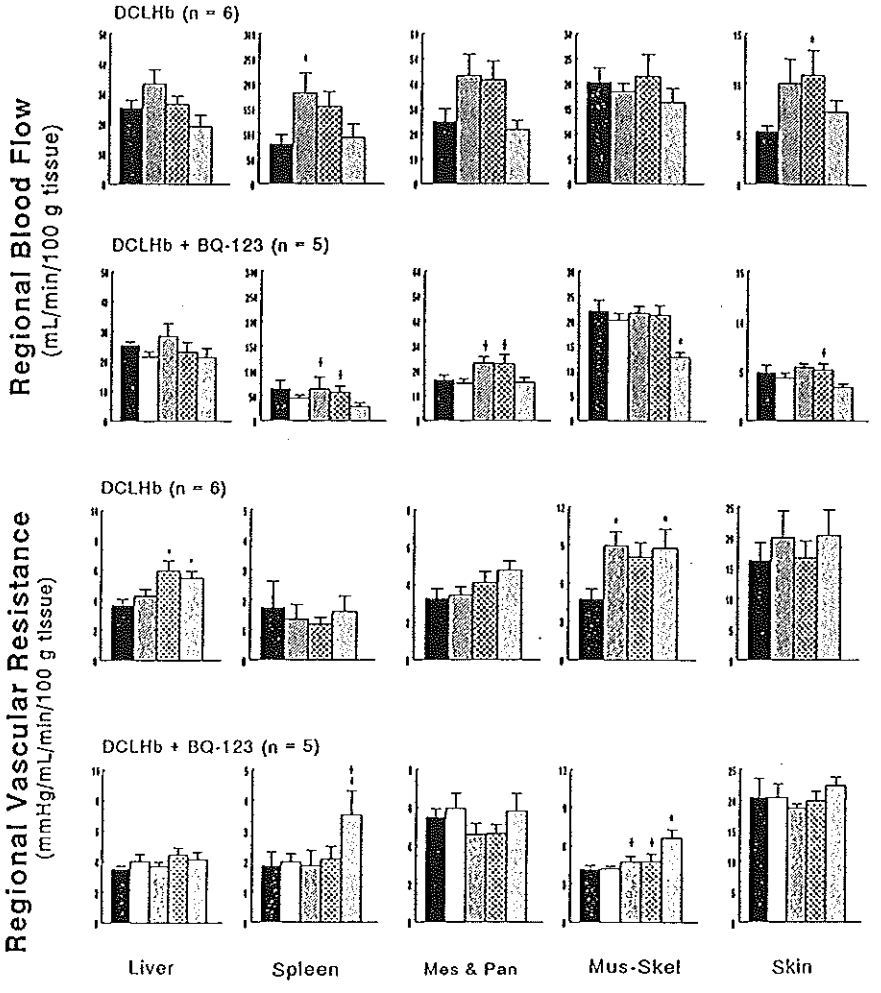

Fig. 4 The effect of DCLHb (400 mg/kg, i.v.) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the liver, spleen, mesentery and pancreas (Mes & Pan) musculoskeletal system (Mus-Skel) and skin before (baseline; solid bars) and at 15 min (hatched bars), 30 min (cross hatched bars) and 60 min (dotted bars) after administration in control (untreated; N = 6) and BQ-123 (5 mg/kg/h, i.v. infusion starting 15 min before the administration of DCLHb (DCLHb); N = 5; hollow bars) treated rats. *Indicates significant difference as compared to baseline and *indicates significant difference as compared to control (DCLHb) group.

Effect of BQ-123 pretreatment on DCLHb induced changes in regional circulation

DCLHb (400 mg/kg, i.v.) increased blood flow to the heart, gastrointestinal tract, spleen and skin, while blood flow to the brain, kidneys, liver, mesentery and pancreas and musculoskeletal system was not altered in control (untreated) rats. In BQ-123 treated rats the blood flow increased only in the gastrointestinal tract but was not altered in the brain, heart, liver, spleen, mesentery and pancreas and skin, and decreased in the kidneys and musculoskeletal system. BQ-123 pretreatment attenuated the increase in blood flow induced by DCLHb to the heart, spleen and skin (Figures 3 and 4).

Infusion of DCLHb increased vascular resistance in the liver and musculoskeletal system, while it was not altered in the brain, heart, gastrointestinal tract, kidneys, spleen, mesentery and pancreas and skin of control rats. In BQ-123 treated rats the vascular resistance was not altered in any region. The increase in vascular resistance induced by DCLHb in the liver and musculoskeletal system was attenuated by BQ-123 pretreatment (Figures 3 and 4).

11.4 Discussion

Haemoglobin based blood substitutes are being developed as promising resuscitative solutions. Early attempts at preparing haemoglobin based blood substitutes involved lysis of human red cells followed by crude separation of haemoglobin from insoluble red cell membranes. These haemoglobin solutions produced significant toxicity. Molecular modifications of haemoglobin have been performed with an attempt to improve its efficacy and safety. Most of the previous studies, irrespective of the stromal contents, have described the haemoglobin solutions as stroma-free haemoglobin or purified stroma free haemoglobin or highly purified stroma free haemoglobin. In the present study we have denoted as SRHb a haemoglobin preparation in which the stromal contents have been partially removed (Zager and Gamelin, 1989) from the soluble haemoglobin. The term stroma-free should ideally mean free of any stromal contents. DCLHb is not only a highly purified, but also a chemically modified, haemoglobin preparation. The phospholipid content, which is a useful indicator of red cell

membrane components (Biessels *et al.*, 1992), was found to be more than 50 ppm in the SRHb, but only 0.2 ppm in the DCLHb.

Haemoglobin solutions have been reported to produce marked hemodynamic changes (Vogel *et al.*, 1986), which have been attributed to improved systemic oxygen delivery (Hauser *et al.*, 1982). However, it is believed that the vascular response to SRHb may be independent of oxygen affinity (Nees *et al.*, 1978). DCLHb, a promising resuscitative solution (Przybelski *et al.*, 1991) produces a pressor response in the rat (Hamilton *et al.*, 1992; Gulati and Rebello, 1994) similar to that of other haemoglobin solutions (Jesch *et al.*, 1982; Rabinovici *et al.*, 1989). Endothelin, nitric oxide (Schultz *et al.*, 1993) and adrenergic (Gulati and Rebello, 1994) mechanisms have been implicated in the pressor effect of DCLHb. Recently, it was demonstrated that DCLHb produces an increase in blood flow to several organs such as heart, spleen, skin and gastrointestinal tract (Sharma and Gulati, 1994). The regional circulatory effects of DCLHb were found to be different from that of norepinephrine, a potent α-adrenoceptor agonist (Sharma and Gulati, 1994). Since an endothelin related mechanism has been implicated in the pressor effect of DCLHb, and endothelin is involved in the regulation of blood circulation (Masaki, 1994), we studied the effect of a specific endothelin-A receptor antagonist, BQ-123, on the regional blood circulatory changes induced by unmodified (SRHb) and modified (DCLHb) haemoglobin solutions. Studies have indicated that BQ-123 *per se* does not produce any significant effect on the cardiovascular system. Intravenous infusion of BQ-123 did not affect systemic hemodynamic parameters and regional blood circulation (Sharma and Gulati, 1995; Douglas *et al.*, 1992).

The regional circulatory and systemic hemodynamic effects of unmodified SRHb were different from those of a purified, modified and stabilized haemoglobin solution, DCLHb. BQ-123, a potent and specific endothelin-A receptor antagonist (Ihara *et al.*, 1992) blocked the pressor effect of DCLHb but not that of SRHb. The pressor effect of DCLHb was more significantly attenuated by BQ-123 than that of SRHb. The increase in total peripheral resistance both by SRHb and DCLHb were blocked by BQ-123 pretreatment. The increase in blood flow to the heart, spleen, gastrointestinal tract and skin induced by DCLHb was blocked by BQ-123 pretreatment. However, the increase in blood flow to the heart induced by SRHb was not

affected by BQ-123 pretreatment. The SRHb induced decrease in blood flow to the kidneys and liver was blocked by BQ-123 pretreatment. It is clear from these results that SRHb and DCLHb produce significant cardiovascular effects, which are mediated through different mechanisms. Since most of the cardiovascular effects of DCLHb and some of those of SRHb are blocked by a specific endothelin-A receptor antagonist, BQ-123, it appears that the cardiovascular effects of DCLHb and of SRHb may be mediated through the endothelin system.

It is well known that endothelin receptors have a wide spread distribution and act through endothelin-A and endothelin-B receptors. Endothelin-A receptors are found both in the periphery and in the central nervous system (Randall, 1991; Gulati and Srimal, 1992). However, the presence of these receptors in the vascular smooth muscles has gained attention and the vascular endothelin system appears to be involved in the regulation of blood flow. Intravenous injection of endothelin into animals causes an initial decrease in blood pressure which is followed by a prolonged pressor response both in anesthetized and chemically denervated rats and conscious rats (King *et al.*, 1990; Rohmeiss *et al.*, 1990). On the other hand, a low dose of endothelin produces hypotension and vasodilatation of isolated perfused arteries *in vitro* (Lippton *et al.*, 1988; Kitazumi *et al.*, 1990). Endothelin-1 produces markedly different effects on blood circulation in different regions (Wright and Fozard, 1988; Le Monnier de Gouville *et al.*, 1990; Gardiner *et al.*, 1990). For example, after endothelin-1 infusion, blood flow decreased in splanchnic areas, while it increased in the skeletal muscles. Endothelin-1 can therefore cause either vasodilatation or vasoconstriction depending on the region (Gardiner *et al.*, 1990).

Endothelin can stimulate the release of vasodilators such as prostacyclin and endothelium derived relaxing factor (EDRF)/nitric oxide (de Nucci *et al.*, 1988). EDRF/nitric oxide has been reported to inhibit the release/synthesis of endothelin-1 (Boulanger and Luscher, 1990). Several factors such as thrombin, arg-vasopressin, angiotensin II, oxyhaemoglobin and hypoxia have been suggested to stimulate the production of endothelin-1 (Miller *et al.*, 1993), which is released on the basal side of the endothelium and acts upon the underlying smooth muscles to produce vasoconstriction (Wagner *et al.*, 1992). DCLHb and SRHb could be releasing endothelin-1, which can lead to the changes in systemic hemodynamics and regional circulation. Endothelin-1, which has a high affinity for endothelin-A receptors, participates in the maintenance of blood

flow of peripheral small resistance vessels (Masaki, 1994). It appears that higher concentrations of endothelin-1 will lead to vasoconstriction due to direct action on endothelin receptors located at the vascular smooth muscles while lower concentrations of endothelin produce vasodilatation by releasing prostacyclin and EDRF/nitric oxide. Phosphoramidon, an inhibitor of pro-endothelin conversion to endothelin, attenuated the pressor effect of DCLHb (Schultz *et al.*, 1993). L-arginine, the substrate for nitric oxide synthesis, and nitroglycerine, an nitric oxide donor, significantly reduced the pressor effect of DCLHb (Schultz *et al.*, 1993). Based on the present findings, DCLHb and SRHb produce different effects on the endothelin system. It could be possible that SRHb affects the endothelin system only in the kidney and liver, while DCLHb affects the endothelin system in most of the organs. It is possible that DCLHb either releases endothelin or increases the sensitivity of vascular endothelin receptors, thereby producing cardiovascular effects.

It was interesting to observe that BQ-123 reversed the SRHb induced decrease in the renal blood flow. Both, endothelin-A and endothelin-B receptors are present in the kidneys. It is well established that endothelin plays a critical role in the renovascular circulation and BQ-123 itself produces a decline in the renovascular resistance, but does not affect the renal blood flow (Pollock and Opgenorth, 1993). The differences in the ability of stroma-free (reduced) haemoglobin and DCLHb to initiate an inflammatory reaction have been demonstrated (Burhop *et al.*, 1992). Infusion of stroma-free (reduced) haemoglobin in sheep has been found to increase the plasma concentrations of C3a and thromboxane B_2, while DCLHb did not elicit any increase in C3a and thromboxane B_2 (Burhop *et al.*, 1992). It has been suggested that the presence of excessive amounts of aminophospholipids in stroma-free (reduced) haemoglobin solution could be responsible for the release of C3a and thromboxane B_2 (Feola *et al.*, 1988). Complement C5b has recently been demonstrated to stimulate the synthesis of endothelin-1 in glomerular epithelial cells (Cybulsky *et al.*, 1993). It could be possible that SRHb initiates an inflammatory reaction which in turn stimulates the synthesis of endothelin-1 in the kidneys leading to decrease in blood flow by SRHb. Stroma reduced and DCLHb produced a significant increase in plasma endothelin-1 concentration. The increase was more marked by SRHb. This favors the

hypothesis that SRHb initiates a more potent inflammatory reaction and thus leads to more marked increase in the plasma endothelin-1 concentration.

It can be concluded that the endothelin-A receptor antagonist BQ-123 could (1) attenuate the pressor effect of DCLHb more significantly than of SRHb, (2) significantly block the effect of DCLHb, but not of SRHb on regional circulation in the heart and (3) block the decrease in blood flow to the kidneys and liver induced by SRHb. These observations tend to suggest that purification, modification and stabilization of haemoglobin produces significant alterations in the cardiovascular effects of haemoglobin which could be mediated through endothelin mechanisms. It is concluded that endothelin mechanisms are involved in the cardiovascular effects of haemoglobin solutions.

11.5 Acknowledgments

The authors would like to thank (1) Dr. P.R. Saxena from Erasmus University, Rotterdam, The Netherlands for providing the software for calculations involved in radioactive microsphere technique, and (2) Baxter Healthcare Corp. for providing financial assistance.

Part 6

Efficacy of diaspirin crosslinked haemoglobin in haemorrhagic shock

Chapter 12

Effect of diaspirin crosslinked haemoglobin, a haemoglobin-based blood substitute, on the regional blood circulation in severely hemorrhaged rats

Summary

Diaspirin crosslinked haemoglobin (DCLHb™), a haemoglobin based blood substitute, has been found to improve regional blood circulation and systemic hemodynamics. We have studied the cardiovascular effects of DCLHb (10% w/v solution, doses of 20%, 50%, and 100% i.v. of shed blood volume, SBV) and Ringer's lactate (300% of SBV) in hemorrhaged rats using a radioactive microsphere technique. Hemorrhage was induced in anaesthetized male Sprague Dawley rats by bleeding them at a rate of approximately 0.5 to 1 ml/min until a mean arterial pressure (MAP) of 35-40 mmHg was achieved. This was maintained for up to 90 min from the onset of hemorrhage to reach a base deficit of less than -12 mmol/l. Hemorrhage significantly decreased MAP, cardiac output (CO), and stroke volume (SV), and increased total peripheral resistance (TPR). Hemorrhage significantly decreased regional blood flow to all the tissues and whole body oxygen consumption. Control rats were administered RL (20% of SBV, i.v.). This volume of RL (vehicle) did not produce any improvements in systemic hemodynamics, regional blood flow or oxygen consumption. DCLHb (20%, 50%, and 100% of SBV) produced significant improvements in systemic hemodynamics and regional blood flow. DCLHb at 20% of SBV improved the oxygen consumption and base deficit from 3.61 ± 0.34 to 4.73 ± 0.47 ml/min and -13.75 ± 0.79 to -10.97 ± 1.28 mmol/l, respectively. At 50% of SBV, DCLHb improved the oxygen consumption and base deficit from 3.49 ± 0.37 to 5.40 ± .060 ml/min and -13.28 ± 0.84 to -8.30 ± 0.89 mmol/l, respectively. At 100% of SBV, DCLHb improved the oxygen consumption and base deficit from 2.62 ± 0.37 to 5.69 ± 0.80 ml/min and -14.02 ± 2.13 to -7.50 ± 1.69 mmol/l, respectively. RL (300% of SBV) when administered at the rate of 1 ml/min for about 40 min, also produced significant improvement in base deficit, oxygen consumption, systemic hemodynamics and regional circulatory parameters. It is concluded that DCLHb in a dose dependent manner produced a significant improvement in oxygen consumption, systemic hemodynamics, and regional blood circulation of hemorrhaged rats.

12.1 Introduction

Prolonged and severe blood loss sufficient to impair oxygen transport leads to hemorrhagic shock which is characterized by failure of compensatory mechanisms, resulting in a loss of homeostasis and death. Hemorrhage is one of the major causes of morbidity and mortality in trauma patients (Faist *et al.*, 1998; Cerra, 1989). In those patients who survive the initial hemorrhagic episode, there is an increased risk of sepsis, multiple organ failure and late death (Ayala *et al.*, 1992; Baue, 1975; Rush, Jr. 1989; Stephan *et al.*, 1987; Cerra, 1989; Mileski *et al.*,

1992). Successful management of hemorrhagic emergencies includes control of ongoing hemorrhage and rapid restoration of intravascular volume to restore tissue perfusion (Wang *et al.*, 1994). Transfusion of homologous blood to enhance oxygen delivery may be life saving (Hankelin *et al.*, 1987; Moore *et al.*, 1992; Shoemaker *et al.*, 1988; Harringer *et al.*, 1992) but is not without problems such as health risks, limitations of supply and uneconomical costs. Homologous blood transfusion may be effective in elective surgeries, but its application in hemorrhagic emergencies is often not feasible. Therefore, there is a need for development of alternative resuscitative solutions.

Haemoglobin based blood substitutes have been proposed to be effective in the treatment of hemorrhagic shock and offer several advantages over non-haemoglobin based resuscitative solutions (Kaplan and Murphy, 1975; Moss *et al.*, 1976; Chang, 1993). DCLHb is a modified haemoglobin solution derived from human erythrocytes. It is prepared by cross-linking haemoglobin between the α-subunits, within the haemoglobin tetramer, by means of a reaction with the diaspirin compound, bis(3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). It is purified by heat pasteurization to inactivate any contaminating viruses and precipitate undesirable proteins (Estep *et al.*, 1989). It possesses biochemical stability and exhibits a greater intravascular retention than unmodified haemoglobin (Hess *et al.*, 1989; Estep *et al.*, 1991). It is slowly degraded in the blood stream and has less accumulation in the tissues, and it is catabolized to low molecular weight compounds which are eliminated through the urine and feces (Estep *et al.*, 1991). DCLHb does not require cross-matching or typing prior to administration, is less viscous than whole blood, and may be better able to carry oxygen through narrowed vessels to ischemic tissues due to the smaller size of the haemoglobin molecule relative to the erythrocytes. DCLHb is devoid of white cells and other blood components which are known to contribute to ischemic tissue injury by releasing cytotoxic products. DCLHb has been found not to elicit any inflammatory reactions in sheep (Burhop *et al.*, 1992) and monkeys (Estep *et al.*, 1992). It does not interfere with the coagulation cascade, or the reticular endothelial system (Przybelski *et al.*, 1991b).

Studies have shown that DCLHb produces significant increases in mean arterial pressure (MAP) in normal anaesthetized (Gulati *et al.*, 1994; Gulati and Rebello, 1994; Gulati and Sharma, 1994) and conscious (Malcolm *et al.*, 1994; Schultz *et al.*, 1993a) rats. In humans, DCLHb produces a dose-dependent increase in MAP without any significant adverse effects or toxicity (Przybelski *et al.*, 1994). This rise in MAP is due to the pharmacological properties of DCLHb solution and not due to volume load or oncotic pressure (Hamilton *et al.*, 1992; Malcolm *et al.*, 1994). In situations simulating clinical settings, DCLHb at 50% of SBV was found to be an effective resuscitative fluid following hemorrhage in rats (Przybelski *et al.*, 1991a; Malcolm *et al.*, 1992). This efficacy was comparable to that of autologous blood and superior to that of isotonic crystalloid solution Ringer's lactate (RL). DCLHb maintained cardiac and renal

functions after partial or complete exchange transfusions (Hess *et al.*, 1989) and restored MAP, cardiac output (CO) and plasma lactate levels (Hess *et al.*, 1993; Hess *et al.*, 1992) after hemorrhage in swine. In rats trained to complete a water alley maze and subjected to hemorrhagic shock, resuscitation with DCLHb did not produce any significant deterioration in their performance (Przybelski *et al.*, 1990). DCLHb at 25% of SBV has been found to decrease base deficit following hemorrhage in rats (Schultz *et al.*, 1993b). Thus DCLHb is an effective resuscitative solution in animal models of hemorrhage.

Previous studies till now have shown the beneficial effects of various doses of DCLHb in restoring the alterations in base deficits, MAP and heart rate (HR) in hemorrhaged rats. No study has yet been performed to determine the effect of DCLHb on oxygen consumption and regional blood circulation in severely hemorrhaged rats. The present study was performed to determine the effect of varying doses of DCLHb on oxygen consumption, base deficit, systemic hemodynamics and regional blood circulation in severely hemorrhaged rats.

12.2 Materials and Methods

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI, USA) weighing 340-400 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, i.p.). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph (Grass Instrument Co., Quincy, MA, USA) through a 7PI preamplifier. The heart rate was recorded through a 7P4B Grass tachograph, triggered from blood pressure signals. The right femoral artery was cannulated to induce controlled hemorrhage. The carotid artery of the right side was exposed and a PE 50 tubing was guided through the common carotid artery into the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P23 DC pressure transducer (Grass Instrument Co., Quincy, Mass.). When the cannula reached the ventricle, the diastolic pressure dropped to zero. In order to keep the blood pO_2, pCO_2 and pH constant, and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a rodent ventilator (Model 683, Harvard Apparatus Inc., South Natick, MA, USA). Arterial blood pO_2, pCO_2 and pH were measured using a pH/blood gas analyzer (ABL330 Radiometer, Copenhagen, Denmark).

Determination of systemic hemodynamics and regional circulation

Systemic hemodynamics and regional blood circulation were determined using the procedure described earlier (Gulati *et al.*, 1994; Sharma and Gulati, 1994). At each measurement, a

thoroughly mixed suspension of approximately 100,000 microspheres (15 ± 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA, USA) in 0.2 ml saline was injected into the left ventricle and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the left femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. All the tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: heart, brain, gastrointestinal tract (GIT), liver, mesentery and pancreas, kidneys, skin and musculoskeletal system. The radioactivity in the standards, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter (Packard Instrument Co., Downers Grove, IL, USA) with preset windows discriminating the isotope energies. The following parameters were calculated: (1) CO [(radioactivity injected x withdrawal rate of arterial blood)/radioactivity in sampled arterial blood], (2) SV [cardiac output/heart rate], (3) total peripheral resistance (TPR) [MAP/CO], (4) regional blood flow [(radioactivity in tissue x withdrawal rate of arterial blood)/radioactivity in sampled arterial blood], and (5) regional vascular resistance [MAP/regional blood flow]. The data were calculated using the computer programs described earlier (Saxena *et al.*, 1980).

Induction of hemorrhage

Hemorrhage was induced by withdrawing blood at the rate of approximately 0.5 to 1 ml/min until a MAP of 35-40 mmHg was reached (Stephan *et al.*, 1987). This was maintained for 90 min from the onset of hemorrhage by additional removal of blood to reach a base deficit of greater than -12 mmol/l (Schultz *et al.*, 1993b).

Determination of hemoximeter values and oxygen consumption

Total haemoglobin concentration, percent oxyhaemoglobin, percent reduced haemoglobin, and oxygen saturation were measured using a hemoximeter (IL482 Co-oximeter system, Instrumentation Laboratory, Lexington, MA, USA). The instrument was set for use of rat blood and the necessary algorithms were applied for species differences. The blood gases and hemoximeter readings were taken at baseline, end of hemorrhage, and 60 min following resuscitation. Oxygen consumption was calculated based on the formula:

Oxygen consumption = CO x 10 x Hgb x 1.36 x (SaO_2-SvO_2) /100,

where CO is cardiac output, Hgb is total haemoglobin, SaO_2 is percent arterial blood oxygen saturated and SvO_2 is percent venous blood oxygen saturated (Bone, 1982).

Resuscitation

The following resuscitation studies were performed (1) RL 20% of SBV (N=12)as vehicle; this will be referred to as the vehicle treated group, (2) RL 300% of SBV (N=10), (3) DCLHb 20% of SBV (N=12), (4) DCLHb 50% of SBV (N=9), and (5) DCLHb 100% of SBV (N=9). Resuscitative solutions were administered intravenously as bolus injections, except for RL 300%

of SBV which was infused at the rate of 1 ml/min for about 40 min. The systemic hemodynamic and regional circulatory effects were determined before the induction of hemorrhage (baseline), following 90 min of hemorrhage and 60 and 120 min after resuscitation.

Drugs

DCLHb was prepared and provided by Baxter Healthcare Corp., Round Lake, IL, USA (Lot No. 94D01AD11-092894V). The physico-chemical characteristics of DCLHb were as follows: Haemoglobin content 10.0 to 10.2 g/dl, methaemoglobin content 1.2 to 1.7 g/dl, p50 at 37°C = 32 to mmHg (defined as the partial pressure of oxygen at which haemoglobin is 50% saturated), osmolality = 290 mOsm/kg, sodium = 140 mEq/l, potassium = 5 mEq/l, phospholipid content <0.2 ppm, endotoxin <0.125 EU/ml and crosslinking between a subunits is 99.9%. RL was purchased from Baxter Healthcare Corp., Deerfield, IL, USA.

Statistics

All data are presented as the mean values ± SEM. MAP (mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by two way analysis of variance followed by Duncan's test. A level of $P < 0.05$ was considered significant.

12.3 Results

Effect of DCLHb on blood gases and hemoximeter values

Hemorrhage significantly decreased the arterial blood pH and pCO_2, and increased pO_2. Administration of vehicle to hemorrhaged rats further decreased arterial blood pH and pCO_2, and increased pO_2. Administration of RL at 300% of SBV or DCLHb at 20%, 50% and 100% of SBV to hemorrhaged rats significantly increased ($p = 0.02$) pH and pCO_2, and decreased ($p = 0.01$) pO_2 (Table 1). Hemorrhage did not produce any alterations in the arterial oxyhaemoglobin, reduced haemoglobin and oxygen saturation. These parameters were not significantly affected following the administration of vehicle, RL or DCLHb. Induction of hemorrhage produced a significant decrease in the arterial total haemoglobin concentration from 15.54 ± 0.13 g% to 10.91 ± 0.24 g% which was not significantly altered following administration of either vehicle, RL or DCLHb.

Effect of DCLHb on oxygen consumption

The basal oxygen consumption in rats was 4.93 ± 0.23 ml/min, however, following hemorrhage, the oxygen consumption significantly ($p = 0.04$) decreased to 3.04 ± 0.20 ml/min. Resuscitation with vehicle further decreased oxygen consumption. On the other hand, resuscitation with RL (300% of SBV) or DCLHb (20, 50 and 100% of SBV) significantly ($p = 0.004$) increased oxygen consumption following hemorrhage. RL (300% of SBV) and DCLHb

when administered in the dose of 50% or 100% of SBV, the oxygen consumption in hemorrhaged rats returned to the baseline levels obtained prior to the induction of hemorrhage (Fig. 1).

Effect of DCLHb on base deficit

The base deficit in normal rats before the induction of hemorrhage was -2.10 ± 0.33 mmol/l. Following hemorrhage the base deficit significantly ($p = 0.03$) decreased to -13.88 ± 0.32 mmol/l. Resuscitation with vehicle further decreased base deficit to -20.76 ± 1.40 mmol/l. On the other hand, resuscitation with RL (300% of SBV) or DCLHb (20, 50 and 100% of SBV) significantly ($p = 0.0001$) increased base deficit following hemorrhage (Fig. 2).

Effect of DCLHb on systemic hemodynamics

The basal value for the HR was 370 ± 20 beats/min, MAP was 81.8 ± 1.55 mmHg, CO was 85.6 ± 2.4 ml/min, stroke volume (SV) was 0.24 ± 0.01 ml, and TPR was 1014 ± 19 mmHg/l/min in normal rats. Induction of hemorrhage significantly ($p < 0.05$) decreased the MAP by >50%, CO by >64%, SV by >61%, and significantly ($p < 0.05$) increased TPR by >32%. The HR was decreased but not significantly following hemorrhage. Resuscitation with vehicle did not produce any improvement in cardiovascular parameters. A decrease in MAP, CO, SV, and increase in TPR was observed following administration of vehicle. A decrease in HR was also observed with vehicle at 60 min of resuscitation. Administration of DCLHb at 20%, 50% and 100% of SBV to hemorrhaged rats significantly ($p = 0.002$) increased the HR by >54%, MAP by >255%, CO by >296%, and SV by >160%. Resuscitation with DCLHb at 20% of SBV did not significantly alter TPR, while administration of RL (300% of SBV) or DCLHb at 50% and 100% of SBV significantly ($p = 0.04$) decreased TPR by >42% as compared to vehicle treated rats (Table 2). Administration of RL at 300% of SBV to hemorrhaged rats significantly ($p = 0.05$) increased the HR by 67%, MAP by 68%, CO by 415%, and SV by 200%. The increase in MAP, CO, and SV following resuscitation with DCLHb (50 and 100% of SBV) were more than that observed following resuscitation with RL (300% of SBV) (Table 2).

TABLE 1 Effect of DCLHb in control (RL 20% of SBV; N=12), RL (300% of SBV; N=10) and DCLHb 20% (N=12), 50% (N=9), and 100% of SBV (N=9) treated hemorrhagic rats in pH, pO_2, and pCO_2.

Parameter	Baseline	Hemorrhage	60 min Resuscitation
pH			
RL 20%	7.42 ± 0.02	7.27 ± 0.02*	7.20 ± 0.03*
RL 300%	7.35 ± 0.01	7.25 ± 0.02*	7.34 ± 0.01#
DCLHb 20%	7.38 ± 0.01	7.27 ± 0.01*	7.31 ± 0.01#
DCLHb 50%	7.41 ± 0.01	7.23 ± 0.04*	7.30 ± 0.02#
DCLHb 100%	7.42 ± 0.02	7.28 ± 0.04*	7.34 ± 0.02#
pO_2			
RL 20%	121.4 ± 7.1	159.1 ± 5.8*	173.0 ± 4.6*
RL 300%	130.3 ± 9.4	162.0 ± 12.1*	143.2 ± 7.4#
DCLHb 20%	119.6 ± 6.3	157.7 ± 7.5*	148.0 ± 7.6#
DCLHb 50%	121.1 ± 2.7	139.9 ± 3.5*	102.1 ± 9.2#
DCLHb 100%	118.7 ± 5.6	145.9 ± 2.2*	106.5 ± 10.1#
pCO_2			
RL 20%	40.0 ± 1.6	27.0 ± 1.2*	19.9 ± 0.6*
RL 300%	32.2 ± 1.8	20.4 ± 2.0*	26.1 ± 2.2#
DCLHb 20%	34.5 ± 1.9	22.5 ± 1.9*	24.8 ± 1.9#
DCLHb 50%	35.5 ± 1.5	26.2 ± 1.9*	34.4 ± 2.7#
DCLHb 100%	33.1 ± 1.3	20.8 ± 1.9*	29.6 ± 3.1#

*Indicates significant ($P < 0.05$) difference as compared to baseline. #Indicates significant ($p < 0.05$) difference as compared to control.

TABLE 2 Effect of DCLHb in control (RL 20% of SBV; N=12), RL (300% of SBV; N=10) and DCLHb 20% (N=12), 50% (N=9), and 100% of SBV (N=9) treated hemorrhagic rats in systemic hemodynamics.

Parameter	Baseline	Hemorrhage	60 min Resuscit.	120 min Resuscit.
Heart rate (beats/min)				
RL 20%	365 ± 11	343 ± 15	248 ± 27*	--
RL 300%	385 ± 7	380 ± 9	415 ± 17#	381 ± 13
DCLHb 20%	382 ± 19	362 ± 23	383 ± 20#	343 ± 20
DCLHb 50%	355 ± 11	349 ± 16	401 ± 9*#	374 ± 6
DCLHb 100%	361 ± 20	348 ± 14	414 ± 11*#	385 ± 28
Mean arterial pressure (mmHg)				
RL 20%	83.5 ± 4.9	35.8 ± 1.4*	21.8 ± 1.6*	--
RL 300%	83.5 ± 3.3	37.3 ± 1.2*	58.4 ± 5.7*#	42.1 ± 5.2*
DCLHb 20%	84.5 ± 3.9	37.2 ± 1.4*	77.5 ± 5.1#	51.8 ± 5.3*
DCLHb 50%	82.7 ± 4.0	36.4 ± 0.7*	104.3 ± 3.8*#	76.5 ± 8.5
DCLHb 100%	75.0 ± 5.2	37.3 ± 0.7*	104.5 ± 6.7*#	92.3 ± 13.4
Cardiac output (ml/min)				
RL 20%	79.9 ± 12.2	24.7 ± 5.1*	12.3 ± 3.1*	--
RL 300%	92.9 ± 7.7	30.2 ± 3.4*	63.3 ± 6.7*#	48.0 ± 5.4*
DCLHb 20%	78.9 ± 4.5	26.8 ± 3.5*	48.7 ± 5.2*#	33.2 ± 5.6*
DCLHb 50%	88.5 ± 5.1	31.6 ± 3.0*	97.4 ± 11.5#	58.0 ± 9.0*
DCLHb 100%	87.7 ± 7.2	30.6 ± 5.4*	152.5 ± 46.4#	56.1 ± 8.8
Stroke volume (ml)				
RL 20%	0.22 ± 0.03	0.07 ± 0.01*	0.05 ± 0.01*	--
RL 300%	0.23 ± 0.02	0.08 ± 0.01*	0.15 ± 0.01*#	0.12 ± 0.01*
DCLHb 20%	0.20 ± 0.01	0.08 ± 0.01*	0.13 ± 0.01*#	0.09 ± 0.01*
DCLHb 50%	0.30 ± 0.03	0.10 ± 0.01*	0.25 ± 0.03#	0.15 ± 0.02*
DCLHb 100%	0.25 ± 0.02	0.09 ± 0.01*	0.37 ± 0.12#	0.15 ± 0.02
Total peripheral resistance (mmHg/l/min)				
RL 20%	1001 ± 82	1538 ± 152*	2127 ± 317*	--
RL 300%	953 ± 82	1355 ± 125*	1021 ± 120*#	925 ± 121
DCLHb 20%	1066 ± 47	1414 ± 74*	1717 ± 124*	1884 ± 210*
DCLHb 50%	992 ± 53	1501 ± 131*	1234 ± 216#	1440 ± 145*
DCLHb 100%	1056 ± 99	1444 ± 187*	976 ± 151#	1762 ± 252*

*Indicates significant ($P < 0.05$) difference as compared to baseline. #Indicates significant ($p < 0.05$) difference as compared to control.

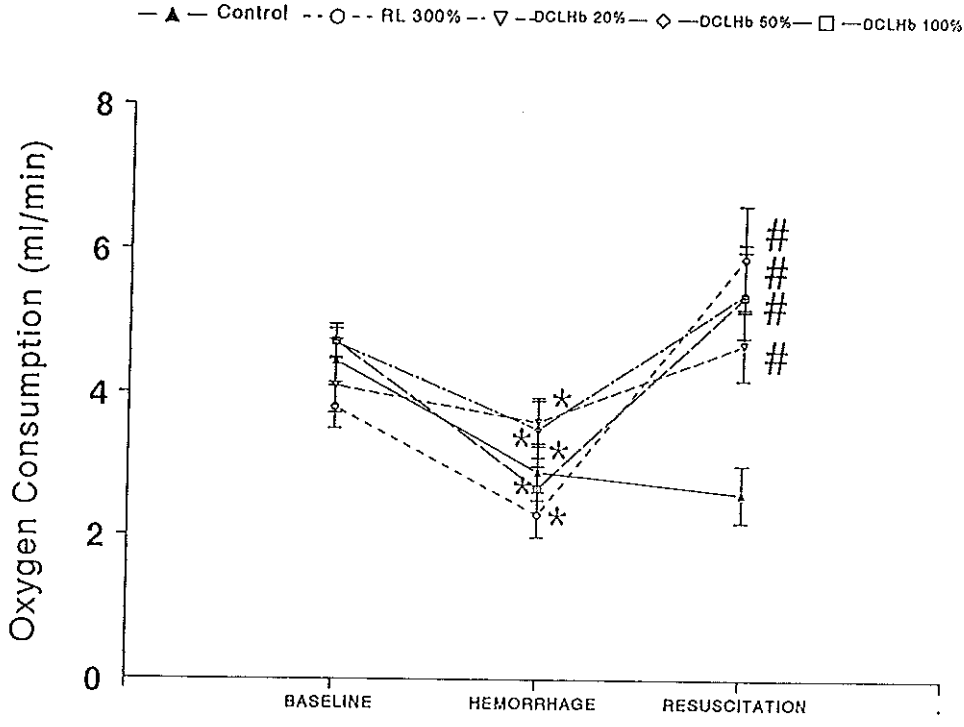

Fig. 1 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on oxygen consumption in hemorrhaged rats at 60 min after resuscitation. *Indicates significant difference as compared to baseline and #indicates significant difference as compared to control group.

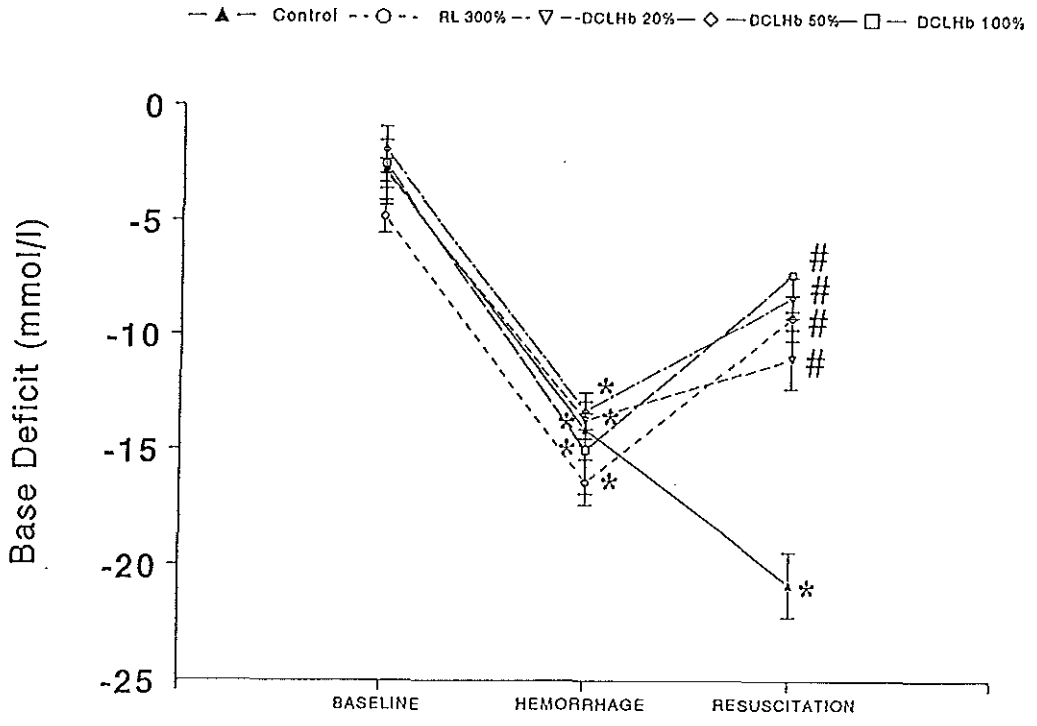

Fig. 2 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on base deficit in hemorrhaged rats at 60 min after resuscitation. *Indicates significant difference as compared to baseline and #indicates significant difference as compared to control group.

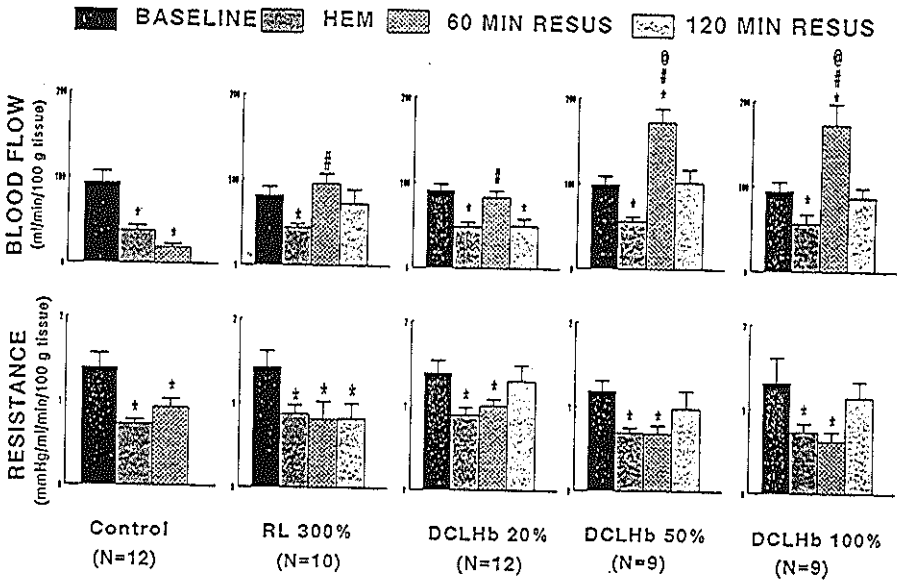

Fig. 3 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the brain of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline, #indicates significant difference as compared to control group and @indicates significant difference as compared to RL group.

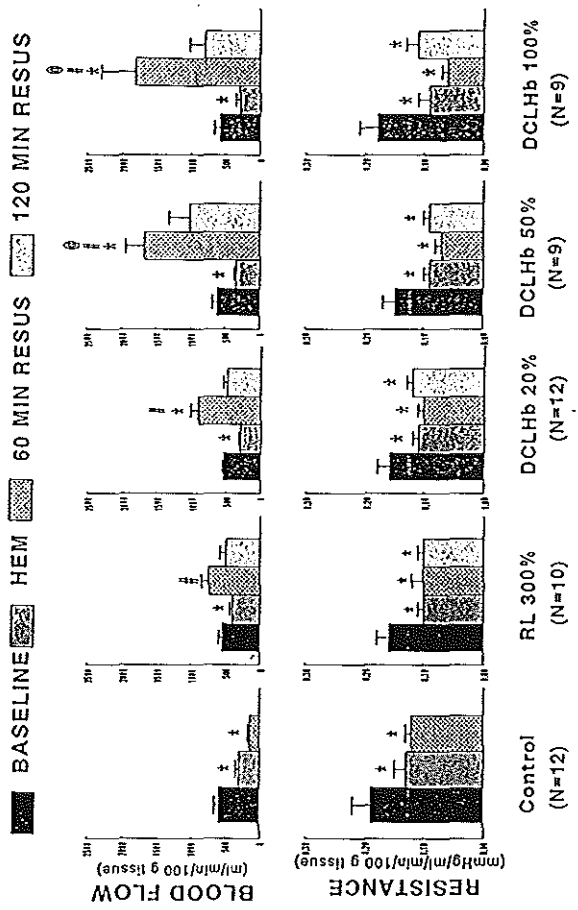

Fig. 4 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the heart of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline, #indicates significant difference as compared to control group and @indicates significant difference as compared to RL group.

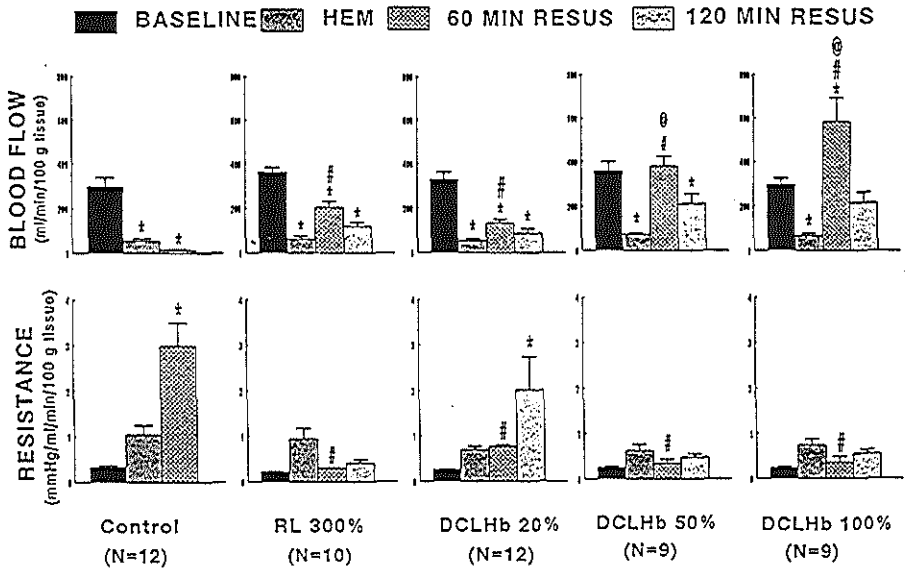

Fig. 5 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the kidneys of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline, #indicates significant difference as compared to control group and @indicates significant difference as compared to RL group.

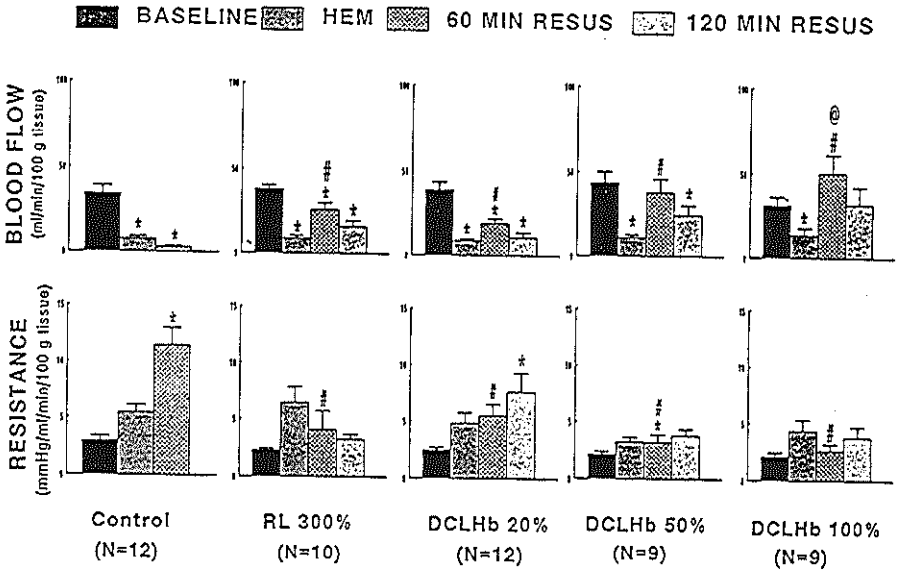

Fig. 6 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the liver of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline, #indicates significant difference as compared to control group and @indicates significant difference as compared to RL group.

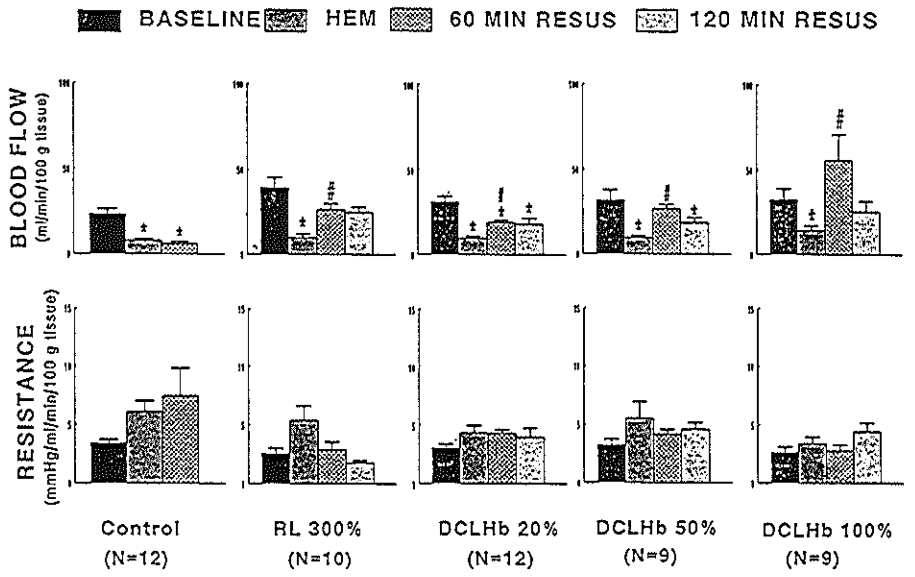

Fig. 7 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the mesentery & pancreas of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline and #indicates significant difference as compared to control group.

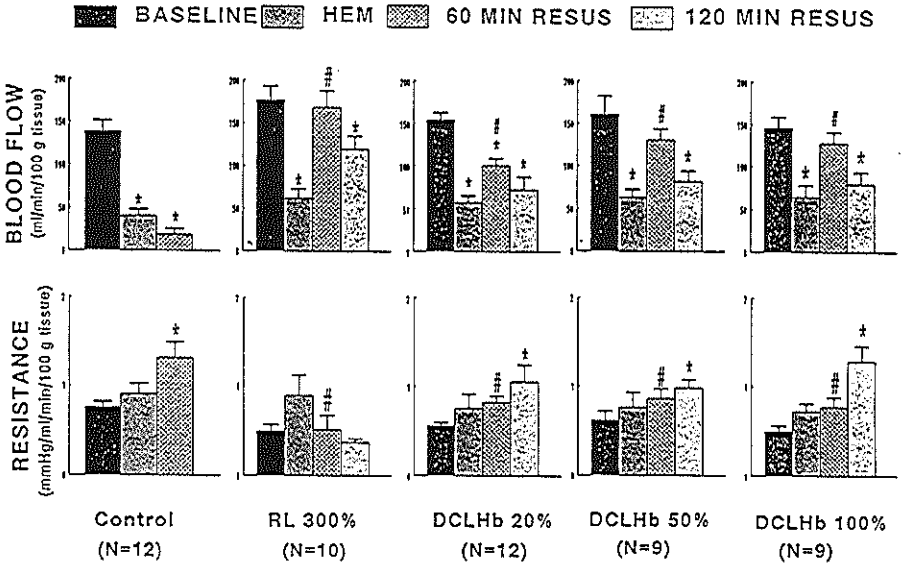

Fig. 8 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the GIT of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline and "indicates significant difference as compared to control group.

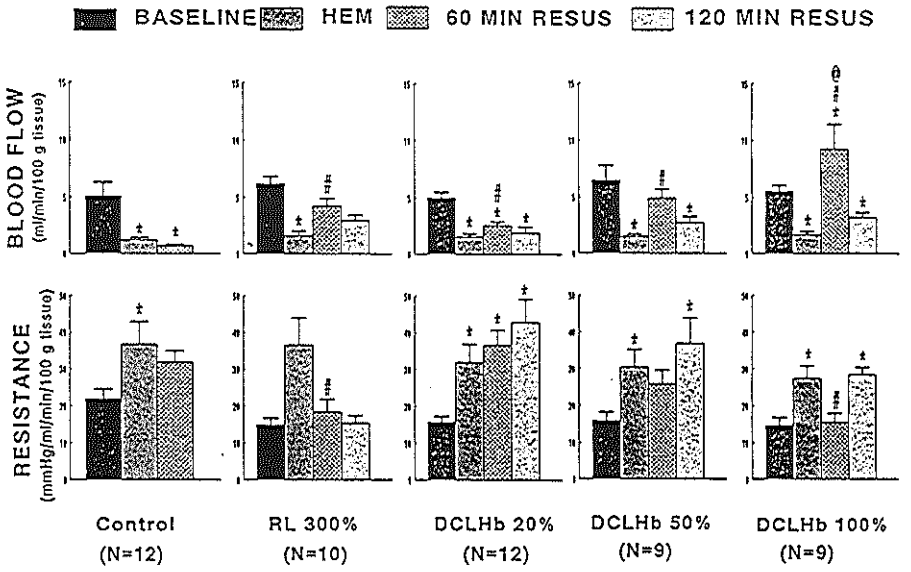

Fig. 9 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the skin of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline, #indicates significant difference as compared to control group and @indicates significant difference as compared to RL group.

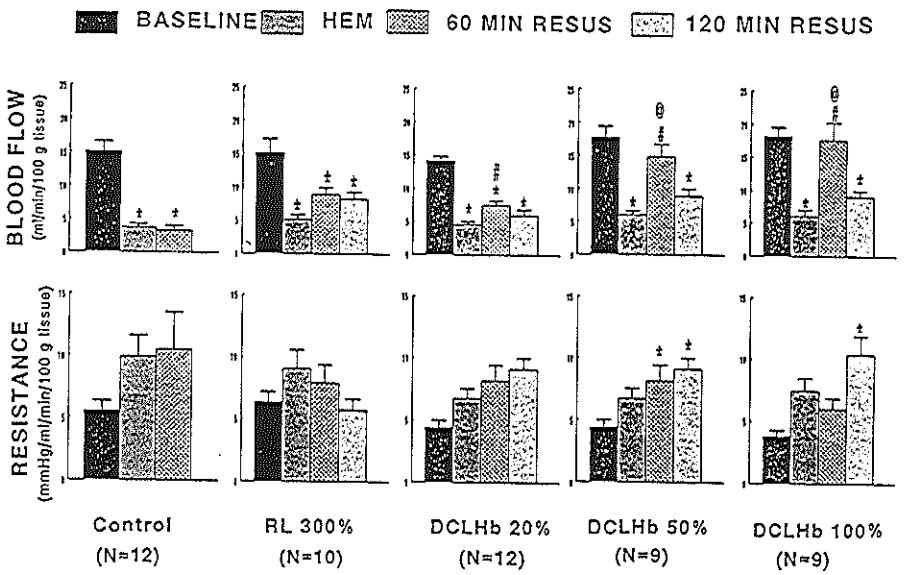

Fig. 10 The effect of DCLHb (20%, 50%, and 100% of SBV) and RL (300% of SBV) on blood flow (ml/min/100 g tissue) and vascular resistance (mmHg/ml/min/100 g tissue) in the musculoskeletal system of hemorrhaged rats before (baseline; solid bars), following hemorrhage (hatched bars), 60 min after resuscitation (cross hatched bars) and 120 min (dotted bars) after resuscitation in control (RL 20% of SBV), and DCLHb (20%, 50%, and 100% of SBV) treated rats. *Indicates significant difference as compared to baseline, #indicates significant difference as compared to control group and @indicates significant difference as compared to RL group.

Effect of DCLHb on regional blood circulation

The basal blood flow to the brain was 94.14 ± 1.22 ml/min/100 g tissue. Hemorrhage significantly ($p = 0.001$) decreased regional blood flow and vascular resistance in the brain. Resuscitation with vehicle did not produce any change in blood flow or vascular resistance in the brain following hemorrhage. Administration of RL (300% of SBV) or DCLHb in the doses of 20%, 50% and 100% of SBV produced an increase in blood flow to the brain 60 min following resuscitation in hemorrhaged rats. The increase in blood flow to the brain following RL or DCLHb administration was significantly ($p = 0.0001$) more than that observed in vehicle treated rats. Vascular resistance was not significantly affected by RL and DCLHb. The increase in blood flow to the brain following resuscitation was significantly more in DCLHb (50 and 100% of SBV) treated as compared to RL (300% of SBV) treated rats (Fig. 3).

In the heart basal blood flow was 562.6 ± 9.58 ml/min/100 g tissue in rats. Induction of hemorrhage produced a significant ($p = 0.002$) decrease in blood flow and vascular resistance in the heart. Administration of vehicle produced no change in blood flow and vascular resistance in the heart of hemorrhaged rats. Resuscitation with RL (300% of SBV) or DCLHb (20%, 50% and 100% of SBV) produced a significant increase in blood flow 60 min after its administration to hemorrhaged rats as compared to vehicle treated rats. The increase in blood flow to the heart following administration of RL or DCLHb to hemorrhaged rats was found to be significantly ($p < 0.0001$) greater than that observed in vehicle treated rats. No significant change was observed in vascular resistance following administration of DCLHb to hemorrhaged rats. The increase in blood flow to the heart following resuscitation was significantly more in DCLHb (50 and 100% of SBV) treated as compared to RL (300% of SBV) treated rats (Fig. 4).

In the kidneys the baseline blood flow was 334.3 ± 15.7 ml/min/100 g tissue in rats. Hemorrhage significantly ($p < 0.0001$) decreased blood flow to the kidneys by >77%, while no change in vascular resistance was observed. Administration of vehicle produced no change in blood flow while an increase in vascular resistance was observed. Resuscitation with RL (300% of SBV) or DCLHb (20%, 50% and 100% of SBV) significantly ($p < 0.0001$) increased blood flow to the kidneys as compared to vehicle treated rats. The increase in vascular resistance observed in vehicle treated rats was not observed in RL (300% of SBV) or DCLHb (50 and 100% of SBV) treated rats. The increase in blood flow to the kidneys following resuscitation was significantly more in DCLHb (50 and 100% of SBV) treated as compared to RL (300% of SBV) treated rats (Fig. 5).

The basal blood flow to the liver was 37.2 ± 1.9 ml/min/100 g tissue and to the mesentery & pancreas was 31.9 ± 2.37 ml/min/100 g tissue. Hemorrhage significantly ($p < 0.0001$) decreased regional blood flow to the liver by >57% and to the mesentery & pancreas by >52% (Figs. 6 and 7). Administration of vehicle produced no change in blood flow to the liver and mesentery & pancreas, while an increase in vascular resistance was observed in the liver (Fig.

6). Resuscitation with RL (300% of SBV) or DCLHb (20%, 50% and 100% of SBV) significantly ($p < 0.0001$) increased blood flow to the liver and mesentery & pancreas as compared to vehicle treated rats. The increase in vascular resistance observed in the liver of RL treated rats was not observed in DCLHb treated rats (Figs. 6 and 7). The increase in blood flow to the liver following resuscitation was significantly more in DCLHb (50 and 100% of SBV) treated as compared to RL (300% of SBV) treated rats (Fig. 6).

In the GIT blood flow was 148.8 ± 3.6 ml/min/100 g tissue in rats. Hemorrhage significantly ($p < 0.0001$) decreased regional blood flow to the GIT by >57%. Resuscitation with vehicle did not produce any change in blood flow or vascular resistance in the GIT following hemorrhage. Administration of RL (300% of SBV) or DCLHb in the doses of 20%, 50% and 100% of SBV produced an increase in blood flow to the GIT 60 min following resuscitation in hemorrhaged rats. The increase in blood flow to the GIT following RL or DCLHb administration was significantly ($p = 0.0001$) greater than that observed in vehicle treated rats. The increase in vascular resistance observed in the GIT of vehicle treated rats at 60 min was not observed in RL or DCLHb treated rats (Fig. 8).

The basal blood flow to the skin was 5.58 ± 0.24 ml/min/100 g tissue and to the musculoskeletal system was 16.82 ± 1.36 ml/min/100 g tissue. Hemorrhage significantly ($p < 0.0001$) decreased regional blood flow to the skin by >69% and to the musculoskeletal system by >67% (Figs. 9 and 10). Administration of vehicle produced no change in blood flow to the skin and musculoskeletal system, while an increase in vascular resistance was observed in the skin (Fig. 9). Resuscitation with RL (300% of SBV) or DCLHb (20%, 50% and 100% of SBV) significantly ($p < 0.0001$) increased blood flow to the skin and musculoskeletal system as compared to vehicle treated rats. The vascular resistance in the skin and musculoskeletal system of DCLHb treated rats was found to be significantly increased. The increase in blood flow to the skin and musculoskeletal system following resuscitation was significantly more in DCLHb (50 and 100% of SBV) treated as compared to RL (300% of SBV) treated rats (Figs. 9 and 10).

12.4 Discussion

Successful prehospital management of severe hemorrhagic shock includes identification and control of bleeding, and to minimization of the time from injury to definitive surgical care by rapid restoration of intravascular volume to restore oxygen delivery and prevent multiple organ failure. Recovery is dependent upon the restoration of oxygen delivery and subsequent oxygen utilization for oxidative phosphorylation by the respiratory enzymes of the mitochondria (Moore *et al.*, 1992; Shoemaker *et al.*, 1988). A rapid restoration of tissue perfusion and oxygenation prevents ischemic tissue damage, multiple organ failure, and improves survival (Moore *et al.*, 1992; Shoemaker *et al.*, 1988).

Base deficit is an extremely sensitive indicator of the severity of volume deficit (Davis *et al.*, 1990; Siegel *et al.*, 1990), metabolic dysfunction (Davis *et al.*, 1990; Siegel *et al.*, 1990), oxygen debt (Dunham *et al.*, 1991), and serum lactate levels (Weiskopf and Fairley, 1982) following hemorrhagic shock. The hemodynamic end-points of resuscitation like MAP, HR, and central venous pressure are often incorrect indicators of adequate perfusion. Oxygen deficit may persist following hemorrhagic shock despite resuscitation and normalization of circulatory function (Siegel *et al.*, 1990; Shoemaker *et al.*, 1982). Thus, the normalization of base deficit is a reliable index for the efficacy of resuscitation following hemorrhagic shock (Rutherford *et al.*, 1992; Davis *et al.*, 1990). The hemorrhagic shock model used in the present study is a well established rodent model of manageable fixed pressure hemorrhage without tissue trauma (Stephan *et al.*, 1987). We have utilized this hemorrhagic shock model and standardized the degree of shock to a base deficit of less than -12 mmol/l (Schultz *et al.*, 1993b).

The present study shows that the vehicle was not effective in increasing oxygen consumption, and reversing base deficit. These suggest persistent oxygen debt and inadequate resuscitation with vehicle. However, when high volume of RL (300% of SBV) was administered as infusion of 1 ml/min for about 40 min, a significant improvement in oxygen consumption was observed. DCLHb (20%, 50%, and 100% of SBV) also produced a significant improvement in oxygen consumption of hemorrhaged rats and restored it to basal values. A consistent finding of an increase in arterial blood pO_2 and a decrease in arterial blood pCO_2 following hemorrhage was observed. This change takes place even in animals kept on positive pressure artificial ventilation. It could be possible that due to artificial ventilation a constant supply of oxygen and carbon dioxide to the pulmonary alveoli is maintained, but subsequent exchange could be responsible for the changes in arterial blood pO_2 and pCO_2 observed in hemorrhaged rats. Hemorrhage produces a significant decrease in systemic and regional blood circulation which will also decrease pulmonary circulation thus allowing more time for the exchange of oxygen and carbon dioxide in the lungs leading to an increase in arterial pO_2 and a decrease in pCO_2. DCLHb at 50% and 100% of SBV reversed base deficit at 60 min after resuscitation, though prehemorrhagic levels were not attained. The reversal of hemorrhage induced base deficits in the present study are comparable with another study in conscious rats using DCLHb 7% w/v solution at 50% and 100% of SBV (Schultz *et al.*, 1993b). However, the conscious rats showed more positive basal values of base deficit (base excess). The lower basal values of base deficit could be due to the anaesthetized state of animals in the present study. Since DCLHb significantly increased the oxygen consumption and reversed the base deficit, it appears that DCLHb is effective in conditions involving hemorrhagic shock. Resuscitation with DCLHb even at 20% and 50% of SBV can restore oxygen delivery and possibly increase tissue perfusion, prevent ischemic tissue damage and multiple organ failure following severe hemorrhage.

Induction of hemorrhage in urethane anesthetized rats has been shown to produce a decrease in MAP, HR and respiratory rate along with a severe decrease in carotid arterial portal vein and abdominal caval blood flow (Guarini *et al.*, 1992). Similar results were obtained in the present study. In the present study vehicle was not effective in improving any of the hemorrhage induced systemic hemodynamic alterations. On the other hand, RL (300% of SBV) produced a significant improvement in systemic hemodynamic parameters of hemorrhaged rats. Resuscitation with DCLHb (20%, 50%, and 100% of SBV) produced a significant pressor effect which was accompanied with significant increases in SV and CO. A significant decrease in TPR was observed when DCLHb was administered in the doses of 50% and 100% of SBV, but due to a marked increase in CO a significant pressor effect was observed in hemorrhaged rats. DCLHb (20%, 50% and 100% of SBV) significantly increased HR in hemorrhaged rats. Similar results have been observed in other studies conducted in hemorrhaged rats using DCLHb 7%, 10%, and 14% w/v solution at 50% and 100% of SBV (Przybelski *et al.*, 1991a; Malcolm *et al.*, 1992). When DCLHb was compared to RL, the improvement in MAP, CO and SV with DCLHb (50 and 100% of SBV) were found to be significantly more marked than RL (300% of SBV).

Most of the resuscitation studies following hemorrhage have been conducted using parameters like survival, MAP, HR, CO and renal functions (Buemi *et al.*, 1993; Bertolini *et al.*, 1989; Maxson *et al.*, 1993; Zingarelli *et al.*, 1994). Only a few studies have been conducted to determine regional blood circulation during hemorrhage and following resuscitation (Wang *et al.*, 1993; Guarini *et al.*, 1992). Besides measuring oxygen consumption, base deficit and systemic hemodynamics, an extensive measurement of regional blood circulation was also performed in the present study.

Hemorrhage produced a significant decrease in blood flow to all the tissues. The decrease in blood flow was less in the brain and heart but was very severe in the kidneys, GIT, portal system and musculoskeletal system. Administration of DCLHb produced a significant improvement in blood flow to all the tissues in a dose dependent manner. This increase in blood flow appeared to be due to a significant increase in CO. The increase in blood flow to all the tissues was highly significant at 60 min after the administration of DCLHb, while at 120 min the blood flow to various tissues decreased but was still higher in some tissues than that observed following hemorrhage. In the present study, vehicle was found to be not effective in restoring the blood flow to any tissue. However, when RL was used in extremely high volumes of 300% of SBV a significant improvement in blood flow to almost all the tissues was observed. These findings are similar to that reported earlier where RL was administered as 400% of SBV and produced a significant improvement in blood flow to the portal system and kidneys (Wang *et al.*, 1993). DCLHb, even in very low volumes (20% to 50%of SBV) was found to be as effective in restoring blood flow to the tissue as extremely high volumes of RL (300 to 400% of SBV).

However, an increased mortality has been reported following use of high volume RL during resuscitation (Wang *et al.*, 1993). DCLHb, besides improving blood flow can also deliver oxygen to the tissues which is not possible with RL. Evidence for the restoration of the decrease in oxygen consumption following hemorrhage by DCLHb has been clearly provided in the present study.

Several types of haemoglobin based blood substitutes have been developed and are in different phases of clinical trials. Studies have demonstrated the efficacy of haemoglobin based blood substitutes in resuscitation following hemorrhage in animals. An ultrapurified, polymerized, bovine haemoglobin solution has been shown to improve the cerebral blood flow when administered to hemorrhaged rats (Waschke *et al.*, 1994). Stroma free polymerized bovine haemoglobin has been found to be effective in restoring the systemic hemodynamic parameters in hemorrhaged dogs (Bosman *et al.*, 1992). Another study using polymerized ultrapurified bovine haemoglobin has shown that it is effective in restoring the cardiovascular parameters following ovarian hemorrhage in a miniature horse (Maxson *et al.*, 1993). A liposome encapsulated haemoglobin preparation consisting of lyophilized powder for use as "instant blood" has also been described (Rabinovici *et al.*, 1995). Several studies have shown the efficacy of DCLHb in hemorrhaged animals (Przybelski *et al.*, 1991a; Schultz *et al.*, 1993b; Malcolm *et al.*, 1992). The present study provides extensive evidence that DCLHb produces significant cardiovascular effects which might be responsible for its efficacy in hemorrhaged rats.

It is known that serious hypotension after hemorrhage is due to a decreased sensitivity of the cardiovascular system to catecholamines (Flint *et al.*, 1984; Baker *et al.*, 1988) and irreversible loss of arteriolar tone (Hutchins *et al.*, 1973). It has been demonstrated that hemorrhagic shock is characterized by a hyporesponsiveness to phenylephrine, an α adrenergic agonist (Zingarelli *et al.*, 1994). DCLHb has been found to increase the sensitivity of vascular α adrenergic receptors (Gulati and Rebello, 1994; Sharma and Gulati, 1995), whether this mechanism is responsible for the efficacy of DCLHb in hemorrhaged rats is not known. DCLHb has also been found to increase endothelin (an endogenous vasoconstrictor substance) levels (Gulati *et al.*, 1996; Gulati *et al.*, 1995) and scavenge nitric oxide (an endogenous vasodilator substance) (Sharma *et al.*, 1995). Therefore, restoration of arteriolar tone could also be achieved as a result of removal of nitric oxide and increases in the concentration of endothelin by DCLHb. Further studies are needed to prove or disprove this hypothesis.

It is concluded that DCLHb is an effective haemoglobin based resuscitative solution in hemorrhaged rats. DCLHb produced a significant improvement in oxygen consumption, base deficit, blood pressure, cardiac output, and regional blood circulation when administered to hemorrhaged rats. It appears that the dose of DCLHb needed to produce maximal effect in hemorrhaged rats is only 50% of SBV, thus DCLHb 20-50% of SBV may be an effective

resuscitative solution in the treatment of acute hemorrhagic shock. Haemoglobin based blood substitutes not only oxygen carrying solutions but produce significant cardiovascular effects and are likely to provide a new armamentarium in the resuscitation of hemorrhagic shock.

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Chapter 13

Role of endothelin and nitric oxide in the resuscitative effect of diaspirin crosslinked hemoglobin, a hemoglobin based blood substitute, following hemorrhage in the rat

Summary

Diaspirin Crosslinked Hemoglobin (DCLHb^{TM}), a hemoglobin based blood substitute, improves regional blood circulation and systemic hemodynamics in normal and hemorrhaged rats. We have studied the modulation of cardiovascular effects of DCLHb by a nitric oxide (NO) synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) and an endothelin-A (ET_A) receptor antagonist FR139317, in severely hemorrhaged rats. Hemorrhage was induced in male urethane anaesthetized rats by bleeding them at a rate of approximately 0.5 to 1 ml/min until a mean arterial pressure (MAP) of 35-40 mmHg was achieved. This MAP was maintained for up to 90 min from the onset of hemorrhage to reach a base deficit of less than -12 mmol/l. Hemorrhage significantly decreased oxygen consumption, arterial blood pH, arterial pCO_2 and increased arterial pO_2. Hemorrhage also produced a significant decrease in MAP, cardiac output (CO) and stroke volume (SV) and increase in total peripheral resistance (TPR). Hemorrhage produced a marked decrease in blood flow to all the tissues and an increase in vascular resistance in the kidneys, skin, portal and musculoskeletal system. Plasma ET-1 and cGMP concentrations were found to be significantly increased following hemorrhage. Control rats were resuscitated with vehicle (Ringer's lactate, RL, 4 ml/kg, i.v.). Resuscitation with vehicle did not produce any improvement in oxygen consumption, base deficit, systemic hemodynamics or regional blood flow of hemorrhaged rats. The vehicle resuscitated rats survived for <70 min following hemorrhage. Resuscitation with DCLHb (400 mg/kg, i.v. in a 10% w/v solution) significantly improved oxygen consumption and base deficit at 60 min following resuscitation. The DCLHb resuscitated rats survived for >120 min following hemorrhage. Resuscitation with DCLHb significantly improved MAP, CO, SV, TPR and blood flow to the heart, gastrointestinal tract, kidneys, brain, skin, portal and musculoskeletal system of hemorrhaged rats in comparison with vehicle treated rats. DCLHb (400 mg/kg, i.v.) significantly increased ET-1 and decreased cGMP concentrations in the blood plasma as compared to vehicle treated rats. Pretreatment with L-NAME (10 mg/kg, i.v.) or FR139317 (4 mg/kg, i.v.) significantly attenuated the DCLHb induced improvement in time of survival, base deficit, systemic hemodynamics and regional blood circulation. It is concluded that DCLHb improves the time of survival, oxygen consumption, systemic hemodynamics and regional blood circulation of severely hemorrhaged rats. Since inhibition NO formation by L-NAME decreased the efficacy of DCLHb, therefore NO removal by DCLHb may not be contributing towards the efficacy of DCLHb in hemorrhaged rats. However, ET antagonist decreased the efficacy of DCLHb, therefore an increase in plasma ET-1 concentration by DCLHb may be contributing towards the efficacy of DCLHb in hemorrhaged

rats. It is concluded that ET mechanism is more important in the beneficial effect of DCLHb in hemorrhaged rats than NO mechanism.

13.1 Introduction

Hemoglobin based blood substitutes are proposed to be effective in the treatment of hemorrhagic shock and offer several advantages over non-hemoglobin based resuscitative solutions (Bunn, 1993; Chang, 1993; Rabinovici *et al.*, 1995). Diaspirin crosslinked hemoglobin (DCLHb™) is a modified hemoglobin solution derived from human erythrocytes. It is prepared by cross-linking purified human hemoglobin between the α-subunits within the hemoglobin tetramer by means of a reaction with the diaspirin compound, bis(3,5-dibromosalicyl) fumarate (Chatterjee *et al.*, 1986). The crosslinked hemoglobin is purified by heat pasteurization to inactivate any contaminating viruses and precipitate any undesirable proteins (Estep *et al.*, 1989). DCLHb possesses biochemical stability and exhibits a greater intravascular retention time than unmodified hemoglobin (Hess *et al.*, 1989; Estep *et al.*, 1991). It is not degraded in the blood stream nor does it accumulate in the tissues where is catabolized to low molecular weight compounds and eliminated through the urine and feces (Estep *et al.*, 1991). DCLHb does not require cross-matching or typing prior to administration, is less viscous than whole blood, and may be better able to carry oxygen through narrowed vessels to ischemic tissues due to the smaller size of the hemoglobin molecule relative to erythrocytes. DCLHb is devoid of white cells and other blood components which are known to contribute to ischemic tissue injury by releasing cytotoxic products. It has been found not to elicit any inflammatory reactions in sheep (Burhop *et al.*, 1992) and monkeys (Estep *et al.*, 1992). It does not interfere with the coagulation cascade or the reticuloendothelial system (Przybelski *et al.*, 1991b).

Studies have shown that DCLHb produces significant increases in MAP in normal anaesthetized (Gulati *et al.*, 1994; Gulati and Rebello, 1994; Gulati *et al.*, 1995b; Sharma and Gulati, 1994) and conscious (Malcolm *et al.*, 1994; Schultz *et al.*, 1993a) rats. In humans, DCLHb produces a dose-dependent increase in MAP with no significant adverse effects or toxicity (Przybelski *et al.*, 1994). The increase in MAP is due to a pharmacological property of DCLHb and not due to volume load or oncotic pressure (Hamilton *et al.*, 1992; Malcolm *et al.*, 1994). DCLHb also produces a significant increase in blood flow to several organs such as the heart, gastrointestinal tract (GIT), portal system and skin. The increase in blood flow to these organs is accompanied with an increase in vascular resistance in the musculoskeletal system (Gulati *et al.*, 1994). In situations simulating clinical settings, DCLHb administered at 50-100% of shed blood volume was found to be an effective resuscitative fluid following hemorrhage in rats (Przybelski *et al.*, 1991a; Malcolm *et al.*, 1992; Powell *et al.*, 1995). The efficacy of DCLHb was comparable to that of autologous blood and superior to that of isotonic crystalloid solution

Ringer's lactate (RL). DCLHb maintained cardiac and renal functions after partial or complete exchange transfusions (Hess *et al.*, 1989) and restored mean arterial pressure (MAP), cardiac output (CO) and plasma lactate levels (Hess *et al.*, 1992; Hess *et al.*, 1993) after hemorrhage in swine. In rats trained to complete a water alley maze and subjected to hemorrhagic shock, resuscitation with DCLHb did not produce any significant degradation in their performance (Przybelski *et al.*, 1990). DCLHb infused at 25% of shed blood volume was found to reduce the base deficit observed following hemorrhage in rats (Schultz *et al.*, 1993b).

Several studies have been performed to determine the mechanisms of DCLHb induced cardiovascular effects. The involvement of adrenergic receptors (Gulati and Rebello, 1994; Sharma and Gulati, 1995; Gulati and Sharma, 1994), endothelin (ET) (Gulati *et al.*, 1995b; Schultz *et al.*, 1993a; Gulati *et al.*, 1996) and nitric oxide (NO) (Schultz *et al.*, 1993a; Katsuyama *et al.*, 1994; Sharma *et al.*, 1995) systems in the cardiovascular responses to DCLHb have been implicated. Plasma ET-1 concentration increases after DCLHb administration (Gulati *et al.*, 1995b) and the cardiovascular effects of DCLHb could be blocked by pretreatment with BQ-123, an ET_A receptor antagonist (Gulati *et al.*, 1996). Plasma cGMP levels in rats are decreased following DCLHb administration and the cardiovascular effects of DCLHb could be blocked by pretreatment with l-arginine, a NO donor (Sharma *et al.*, 1995). These studies were performed in normal animals. No comparable study has been conducted to determine the mechanisms involved in the action of DCLHb in hemorrhaged rats. The experiments described in this paper were conducted to determine the role of ET and NO in the cardiovascular actions of DCLHb in hemorrhaged rats. Studies were performed to determine the modulation of cardiovascular effects of DCLHb in hemorrhaged rats by L-NAME, a nitric oxide synthase (NOS) inhibitor and FR139317 ((R)2-[(R)-2[(S)-2[[1-hexahydro-1H-azepinyl)]-carbonyl]amino-4-methyl-pentanoyl]amino-3[3-(1-methyl-1H-indolyl)]propinonyl]amino-3-(2-pyridyl)propionic acid), a specific ET_A receptor antagonist. Biochemical studies were also performed to determine the effect of hemorrhage and of DCLHb on plasma ET and cGMP levels.

13.2 Materials and Methods

Animals and surgical preparations

Male Sprague-Dawley rats (Sasco-King Animal Co. Oregon, WI, USA) weighing 330-370 g were used in the study. Rats were anesthetized with urethane (1.5 g/kg, i.p.). The left femoral vein was cannulated (PE 50 tubing) for drug administration. The left femoral artery was cannulated (PE 50 tubing) and connected to a Gould P23 ID pressure transducer for recording the blood pressure on a Grass P7D polygraph (Grass Instrument Co., Quincy, MA, USA) through a 7PI preamplifier, and also for blood withdrawal using a withdrawal pump (Model 22, Harvard Apparatus, South Natick, MA, USA). The heart rate was recorded through a 7P4B Grass

tachograph (Grass Instrument Co., Quincy, MA, USA) triggered from blood pressure signals. The right femoral artery was cannulated to induce controlled hemorrhage. The carotid artery of the right side was exposed and a PE 50 tubing was guided through the common carotid artery into the left ventricle. The presence of the cannula in the left ventricle was confirmed by recording the pressure on the Grass polygraph using the Statham P23 DC pressure transducer (Grass Instrument Co., Quincy, MA, USA). When the cannula reached the ventricle, the diastolic pressure dropped to zero. In order to keep the blood pO_2, pCO_2 and pH constant, and to avoid the effect of respiration on blood pressure and heart rate, animals were kept on constant rate artificial respiration by inserting an endotracheal cannula connected to a rodent ventilator (Model 683, Harvard Apparatus Inc., South Natick, MA, USA). Arterial blood pO_2, pCO_2 and pH were measured using a pH/blood gas analyzer (ABL330 Radiometer, Copenhagen, Denmark).

Determination of systemic hemodynamics and regional circulation

Systemic hemodynamics and regional blood circulation were determined using the procedure described earlier (Gulati *et al.*, 1994; Sharma and Gulati, 1994). At each measurement, a thoroughly mixed suspension of approximately 100,000 microspheres (15 $\pm$ 1 μm diameter) labeled with ^{46}Sc (Scandium), ^{113}Sn (Tin), ^{141}Ce (Cerium), ^{95}Nb (Niobium) or ^{103}Ru (Ruthenium) (New England Nuclear Corporation, Boston, MA, USA) in 0.2 ml saline was injected into the left ventricle and flushed with 0.4 ml saline over a 15 sec period. In order to calculate the blood flow, arterial blood was withdrawn at a rate of 0.5 ml/min through the left femoral artery. Blood was withdrawn for 90 sec starting about 5-10 sec before the microsphere injection. The animals were kept on the respirator and observed until death occurred. The time of death was noted to calculate the survival time (min) from the induction of hemorrhage. All the tissues and organs were dissected out, weighed and placed in vials. The following tissues were studied: heart, brain, gastrointestinal tract (GIT), liver, mesentery and pancreas, kidneys, skin and musculoskeletal system. The radioactivity in the standards, the blood samples and the tissue samples were counted in a Packard Minaxi Auto-Gamma 5000 series gamma counter (Packard Instrument Co., Downers Grove, IL, USA) with preset windows discriminating the isotope energies. The following parameters were calculated: (1) cardiac output (CO) [(radioactivity injected x withdrawal rate of arterial blood)/radioactivity in sampled arterial blood], (2) stroke volume (SV) [CO/heart rate], (3) total peripheral resistance (TPR) [MAP/CO], (4) regional blood flow [(radioactivity in tissue x withdrawal rate of arterial blood)/radioactivity in sampled arterial blood], and (5) regional vascular resistance [MAP/regional blood flow]. The data were calculated using the computer programs described earlier (Saxena *et al.*, 1980).

Induction of hemorrhage

Hemorrhage was induced by withdrawing blood at a rate of approximately 0.5 to 1 ml/min until a MAP of 35-40 mmHg was reached (Stephan *et al.*, 1987). This MAP was maintained for

90 min from the onset of hemorrhage to reach a base deficit of greater than -12 mmol/l (Schultz *et al.*, 1993b).

Determination of hemoximeter values and oxygen consumption

Total hemoglobin concentration, percent oxyhemoglobin, percent reduced hemoglobin, and oxygen saturation were measured using a hemoximeter (IL482 Co-oximeter system, Instrumentation Laboratory, Lexington, MA, USA). The blood gas and hemoximeter readings were taken at baseline, end of hemorrhage, and 60 min following resuscitation. Oxygen consumption was calculated based on the formula:

Oxygen consumption = CO x 10 x Hgb x 1.36 x (SaO_2-SvO_2) /100

where CO is cardiac output, Hgb is total hemoglobin, SaO_2 is percent arterial blood oxygen saturated and SvO_2 is percent venous blood oxygen saturated (Bone, 1982).

Resuscitation

The following resuscitation studies were performed (1) vehicle (RL 4 ml/kg, i.v.), (2) DCLHb (400 mg/kg, i.v. in a volume of 4 ml/kg), (3) L-NAME (10 mg/kg, i.v.) + DCLHb (400 mg/kg, i.v.), and (4) FR139317 (4 mg/kg, i.v.) + DCLHb (400 mg/kg, i.v.). Both L-NAME and FR139317 were administered 15 min prior to resuscitation with DCLHb. The systemic hemodynamic and regional circulatory effects were determined before the induction of hemorrhage (baseline), following 90 min of hemorrhage and 60 and 120 min after resuscitation. The dose of DCLHb was selected on the basis of previous studies showing this dose produces a near maximal pressor response and is effective in resuscitation following hemorrhage (Gulati and Rebello, 1994; Hamilton *et al.*, 1992; Schultz *et al.*, 1993b).

Measurement of ET-1 concentration

Blood samples were collected in plastic tubes containing ethylenediaminetetraacetate (EDTA, 1 mg/ml) and aprotinin (500 kIU/ml), centrifuged (15 min, 4°C, 1,000 x g) and the plasma collected. Plasma was mixed with an equal volume of 20% acetic acid, centrifuged (15 min, 4°C, 1,000 x g) and the supernatant was collected for subsequent assay. The radioimmunoassay for ET-1 was performed as described earlier (Gulati *et al.*, 1995b). All reagents were kept on ice and all procedures were performed at 4°C. The SEP-columns were activated by successively washing with 3 ml methanol, 2 ml water and 2 ml of 10% acetic acid solution. The samples were slowly applied on the column and then allowed to flow through the column at the rate determined by gravity. The columns were washed with 2 ml of 10% acetic acid followed by 3 ml of ethyl acetate. ET was eluted with 1.5 ml of elution buffer (1 : 4; 0.05 M ammonium bicarbonate solution : methanol) into polypropylene tubes. The eluate was evaporated to dryness under a gentle stream of nitrogen gas. The recovery was found to be 92% by this method. The radioimmunoassay was performed using antibodies specific for ET-1. This antibody has only 7% cross reactivity with ET-2 or ET-3. The incubation mixture for the radioimmunoassay consisted of 100 µl of standards or samples and 100 µl of rabbit anti-ET-1

serum. The tubes were vortexed and incubated overnight at 4°C. The next day, [^{125}I] ET-1 (100 µl) was added and the mixture vortexed and incubated for 24 hours. On day 3, goat anti-rabbit IgG serum (100 µl) and normal rabbit serum (100 µl) were added to all the tubes, vortexed and incubated for 2 h at room temperature. Radioimmunoassay buffer (500 µl) was added, the tubes were vortexed and then centrifuged at 1,700 g for 20 min. The supernatant was carefully aspirated and the radioactivity in the pellet was determined using a Packard Gamma Counter (Model Cobra 5005, Packard Instruments, Downers Grove, IL, USA). The concentration of ET-1 was determined from the standard plots of % total (B)/total-nonspecific (B_o) binding (B/B_o) versus log dose (pg) and expressed as pg/ml in plasma and pg/g wet weight in tissues. ET-1 estimations were performed in (1) control (untreated), (2) hemorrhaged, (3) control (RL 4 ml/kg, i.v.), and (4) DCLHb (400 mg/kg, i.v.) treated rats.

Measurement of cGMP concentration

The plasma samples were assayed for cGMP as described earlier (Harper and Brooker, 1975). Blood was collected in plastic tubes containing EDTA (1 mg/ml) and aprotinin (500 kIU/ml) and centrifuged (15 min, 4°C, 1,000 x g) and plasma was separated and assayed for cGMP. Water saturated ether (2.5 ml) was mixed with 0.5 ml of plasma and the aqueous layer was extracted. The procedure was repeated 3 times and then the combined extracts were evaporated to dryness under nitrogen. The residue was dissolved in 50 mM sodium acetate buffer (pH 4.75). Aliquots (0.5 ml) were taken from each sample after ether extraction for determination of cGMP. Acetylation was performed directly on each sample at room temperature by adding 0.01 ml of triethylamine followed immediately with the addition of 0.5 ml of acetic anhydride to minimize the time of exposure of the sample to basic conditions. Cyclic GMP immunoassay was performed in 50 mM sodium acetate buffer (pH 4.75) using a cGMP RIA kit (Advanced Magnetics Inc., Cambridge, USA). The incubation mixture for RIA consisted of 100 µl of standard or sample, 100 µl of tracer and 100 µl of anti serum. The tubes were vortexed and incubated overnight at 4°C. The following day, magnetic goat anti-rabbit IgG (500 µl) was added and incubated for 20 min at room temperature. The tubes were then placed in a magnetic separation unit for 10 min. The supernatant was decanted and the radioactivity was determined using a Gamma counter (Model Cobra 5005; Packard Instruments, Downers Grove, IL, USA) and the data calculated as described in the previous section. The concentration of cGMP was expressed as pmoles/ml of plasma. cGMP estimations were performed in (1) control (untreated), (2) hemorrhaged, (3) control (RL 4 ml/kg, i.v.), and (4) DCLHb (400 mg/kg, i.v.) treated rats.

Drugs

DCLHb (10% w/v solution; lot ??) was prepared and provided by Baxter Healthcare Corp., Round Lake, IL, USA. The physico-chemical characteristics of DCLHb were as follows: Hemoglobin content 10.0 to 10.2 g/dl, methemoglobin content 0.7 to 1.2 g/dl, p50 at 37°C = 32

mmHg (defined as the partial pressure of oxygen at which hemoglobin is 50% saturated), osmolality = 290 mOsm/kg, sodium = 140 mEq/l, potassium = 5 mEq/l, phospholipid contents < 0.2 ppm, endotoxin < 0.125 EU/ml and crosslinking between the α subunits is 99.9%. L-NAME was purchased from Sigma Chemical Co., St. Louis, MO, USA. FR139317 (Fujisawa Pharmaceutical Co.) was provided by Dr. K. Buchan, Glaxo Research Group, England, UK. L-NAME and FR139317 were freshly prepared on each day of the experiment. [^{125}I]Tyr^{13}-ET-1 (human, porcine) was purchased from DuPont NEN Research Products, Wilmington, DE. SEP-Columns (200 mg), rabbit anti-ET-1 (porcine, human) serum, normal rabbit serum and goat anti-rabbit IgG serum were purchased from Peninsula Laboratories Inc., Belmont, CA. The RIA buffer for ET-1 consisted of 19 mM NaH_2PO_4, 81 mM Na_2HPO_4, 0.05 M NaCl, 0.1 % bovine serum albumin, 0.1 % TritonX-100 and 0.01 % NaN_3. Ringer's lactate was purchased from Baxter Healthcare Corp., Deerfield, IL, USA.

Statistics

All data are presented as the mean values ± SEM. MAP (mmHg) was calculated using the formula [(Systolic BP - Diastolic BP) / 3] + Diastolic BP. Heart rate was recorded as beats/min. Data were analyzed by two way analysis of variance followed by Duncan's multiple range test. A level of $P < 0.05$ was considered significant.

13.3 Results

Effect on blood gases and hemoximeter values

Hemorrhage significantly decreased ($p = 0.01$) arterial blood pH and pCO_2, and increased ($p = 0.03$) pO_2. Resuscitation with vehicle (RL, 4 ml/kg, i.v.) further decreased arterial blood pH and pCO_2, and increased pO_2. However, resuscitation with DCLHb significantly increased ($p = 0.01$) pH and pCO_2, and decreased ($p = 0.002$) pO_2, when compared to vehicle resuscitated rats. Pretreatment with L-NAME or FR139317 did not alter the DCLHb induced changes in pH, pCO_2 and pO_2 during resuscitation (Table 1). Hemorrhage did not produce any alterations in the arterial oxyhemoglobin content, reduced hemoglobin concentration or oxygen saturation value. These parameters were not significantly altered by resuscitation with vehicle and DCLHb, L-NAME + DCLHb or FR139317 + DCLHb. Induction of hemorrhage produced a significant decrease in the arterial total hemoglobin content from 15.12 ± 0.34 g% to 11.66 ± 0.63 g% which was not affected by resuscitation with vehicle, DCLHb, L-NAME + DCLHb or FR139317 + DCLHb.

Effect on mortality

The vehicle (RL, 4 ml/kg, i.v.) resuscitated rats showed 100% mortality by 90 min following resuscitation. The DCLHb treated rats showed 100% survival for >120 min following

resuscitation. However, when DCLHb was administered to L-NAME or FR139317 pretreated rats a 50% mortality by 120 min was observed following resuscitation.

Effect on oxygen consumption

The basal oxygen consumption in normal rats ranged from 4.53 to 4.78 ml/min in various groups. Following hemorrhage the oxygen consumption significantly decreased ($p = 0.02$) and ranged from 2.57 to 3.61 ml/min in various groups. Resuscitation with vehicle further decreased the oxygen consumption. Resuscitation with DCLHb significantly increased ($p = 0.002$) the oxygen consumption to basal levels when compared to control vehicle resuscitated rats (Fig. 1). Pretreatment with L-NAME or FR139317 significantly attenuated ($p = 0.0004$) the DCLHb induced increase in oxygen consumption (Fig. 1).

Effect on base deficit

The base deficit in normal rats ranged from -1.18 to -2.78 mmol/l in various groups. Following hemorrhage the base deficit significantly decreased ($p = 0.0001$) and ranged from -13.28 to -14.12 mmol/l in various groups. Resuscitation with vehicle further decreased base deficit, while resuscitation with DCLHb significantly increased ($p = 0.0001$) base deficit compared to vehicle resuscitated rats (Fig. 2). Pretreatment with L-NAME and FR139317 significantly attenuated ($p = 0.0001$) the DCLHb induced increase in base deficit (Fig. 2).

Effect on systemic hemodynamics

Hemorrhage significantly decreased ($p = 0.02$) MAP, CO and SV, and significantly increased ($p = 0.04$) TPR. The HR was not significantly affected following hemorrhage. A further decrease in HR, MAP, CO and SV, and increase in TPR were observed following resuscitation with vehicle. Resuscitation with DCLHb significantly increased ($p = 0.01$) HR by >61%, MAP by >237%, CO by >396%, and SV by >175%, when compared with vehicle resuscitated rats. Resuscitation with DCLHb did not affect TPR. Pretreatment with L-NAME significantly attenuated the DCLHb induced increase ($p = 0.02$) in MAP, along with an insignificant decrease in CO and SV. Pretreatment with L-NAME did not alter HR and TPR. Pretreatment with FR139317 significantly attenuated the DCLHb induced increase in MAP ($p = 0.0005$), and TPR ($p = 0.03$). Pretreatment with FR139317 did not alter HR, CO and SV in DCLHb resuscitated rats (Table 2).

TABLE 1 Effect of DCLHb (400 mg/kg, i.v.; N=8) in control (RL 4 ml/kg, i.v.; N=7), L-NAME (10 mg/kg, i.v.; N=8) and FR139317 (FR; 4 mg/kg, i.v.; N=6) pretreated hemorrhagic rats on pH, pO_2, and pCO_2.

Parameter	Baseline	Hemorrhage	60 min Resuscitation
pH			
Control	7.38 ± 0.02	7.26 ± 0.02*	7.21 ± 0.03*
DCLHb	7.41 ± 0.01	7.27 ± 0.01*	7.30 ± 0.01^{+}
L-NAME + DCLHb	7.42 ± 0.01	7.28 ± 0.03*	7.33 ± 0.02
FR + DCLHb	7.39 ± 0.02	7.24 ± 0.04*	7.34 ± 0.02
pO_2			
Control	123.4 ± 6.4	146.7 ± 6.8*	187.3 ± 7.6*
DCLHb	120.6 ± 7.1	158.3 ± 8.1*	141.0 ± 8.6^{+}
L-NAME + DCLHb	118.1 ± 5.6	153.2 ± 7.3*	147.9 ± 7.5
FR + DCLHb	124.4 ± 7.8	161.4 ± 7.7*	138.6 ± 8.3
pCO_2			
Control	38.4 ± 2.6	28.0 ± 2.2*	20.9 ± 2.1*
DCLHb	38.8 ± 2.9	23.4 ± 3.4*	27.8 ± 2.3^{+}
L-NAME + DCLHb	41.6 ± 1.7	26.9 ± 2.7*	29.9 ± 2.1
FR + DCLHb	35.5 ± 3.1	22.6 ± 2.9*	31.6 ± 2.7

*Indicates significant ($p < 0.05$) difference as compared to baseline. $^{+}$Indicates significant ($p < 0.05$) difference as compared to control.

TABLE 2 Effect of DCLHb (400 mg/kg, i.v.; N=8) in control (RL 4 ml/kg, i.v.; N=7), L-NAME (10 mg/kg, i.v.; N=8) and FR139317 (FR; 4 mg/kg, i.v.; N=6) pretreated hemorrhagic rats on systemic hemodynamics.

Parameter	Baseline	Hemorrhage	60 min Resus.	120 min Resus.
Heart rate (beats/min)				
Control	385 ± 20	349 ± 18	235 ± 31*	--
DCLHb	399 ± 37	361 ± 31	379 ± 29^{+}	354 ± 29
L-NAME + DCLHb	371 ± 22	346 ± 16	328 ± 23^{+}	255 ± 37^{b}
FR + DCLHb	402 ± 26	394 ± 22	359 ± 27^{+}	305 ± 35*§
Mean arterial pressure (mmHg)				
Control	91.2 ± 5.6	36.5 ± 2.3*	22.5 ± 1.6*	--
DCLHb	87.6 ± 5.4	37.5 ± 2.4*	75.8 ± 6.6^{+}	55.2 ± 6.7
L-NAME + DCLHb	82.0 ± 6.5	39.9 ± 1.4*	50.9 ± 6.5*$^{+@}$	31.9 ± 4.8^{b}
FR + DCLHb	78.7 ± 4.7	35.8 ± 2.1*	36.2 ± 3.8*$^{+@}$	33.4 ± 4.4*§
Cardiac output (ml/min)				
Control	78.8 ± 20.0	17.7 ± 5.0*	8.3 ± 1.4*	--
DCLHb	66.9 ± 5.6	23.3 ± 5.0*	41.2 ± 5.1*$^{+}$	31.4 ± 6.9*
L-NAME + DCLHb	85.9 ± 8.5	31.1 ± 1.9*	28.2 ± 4.2*$^{+}$	20.6 ± 6.4*^{b}
FR + DCLHb	70.4 ± 6.9	27.7 ± 3.3*	42.1 ± 6.3*$^{+}$	39.6 ± 8.1*§
Stroke volume (ml)				
Control	0.20 ± 0.05	0.06 ± 0.02*	0.04 ± 0.01*	--
DCLHb	0.17 ± 0.01	0.07 ± 0.02*	0.11 ± 0.02*$^{+}$	0.09 ± 0.01*
L-NAME + DCLHb	0.22 ± 0.02	0.09 ± 0.01*	0.08 ± 0.01*$^{+}$	0.08 ± 0.02*^{b}
FR + DCLHb	0.18 ± 0.01	0.06 ± 0.01*	0.10 ± 0.03*$^{+}$	0.13 ± 0.02§
Total peripheral resistance (mmHg/l/min)				
Control	1107 ± 121	1689 ± 215*	1961 ± 214*	--
DCLHb	1164 ± 142	1612 ± 101*	1919 ± 133*	1981 ± 183*
L-NAME + DCLHb	1039 ± 129	1481 ± 119*	1921 ± 212*	2061 ± 665^{b}
FR + DCLHb	1193 ± 98	1736 ± 181*	1107 ± 266$^{+@}$	1094 ± 227§

*Indicates significant ($p < 0.05$) difference as compared to baseline. $^{+}$Indicates significant ($p < 0.05$) difference as compared to control. $^{@}$Indicates significant ($p < 0.05$) difference as compared to DCLHb treatment. ^{b}Indicates N=4. §Indicates N=3.

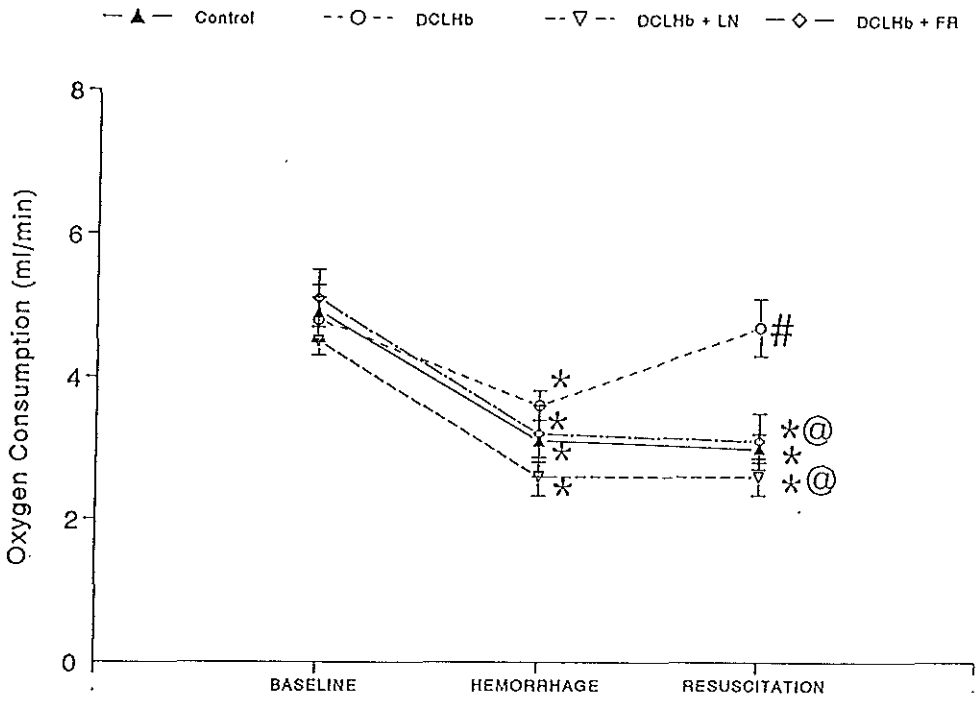

Fig. 1 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (LN, 10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (FR, 4 mg/kg, i.v.) pretreated rats on oxygen consumption before and after hemorrhage and at 60 min after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

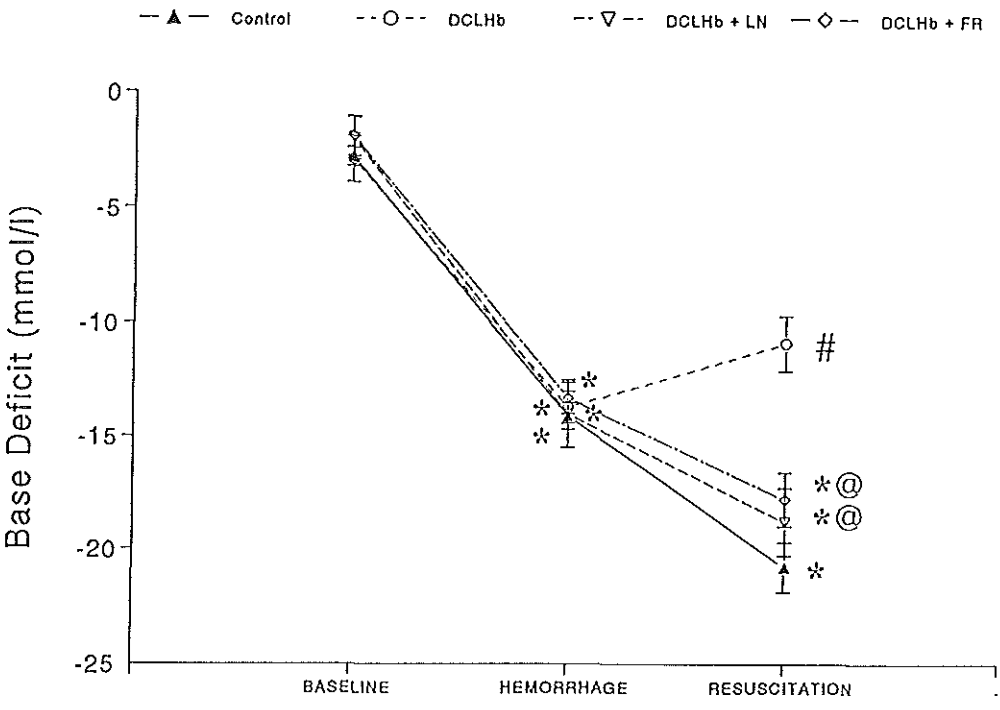

Fig. 2 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (LN, 10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (FR, 4 mg/kg, i.v.) pretreated rats on base deficit before and after hemorrhage and at 60 min after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

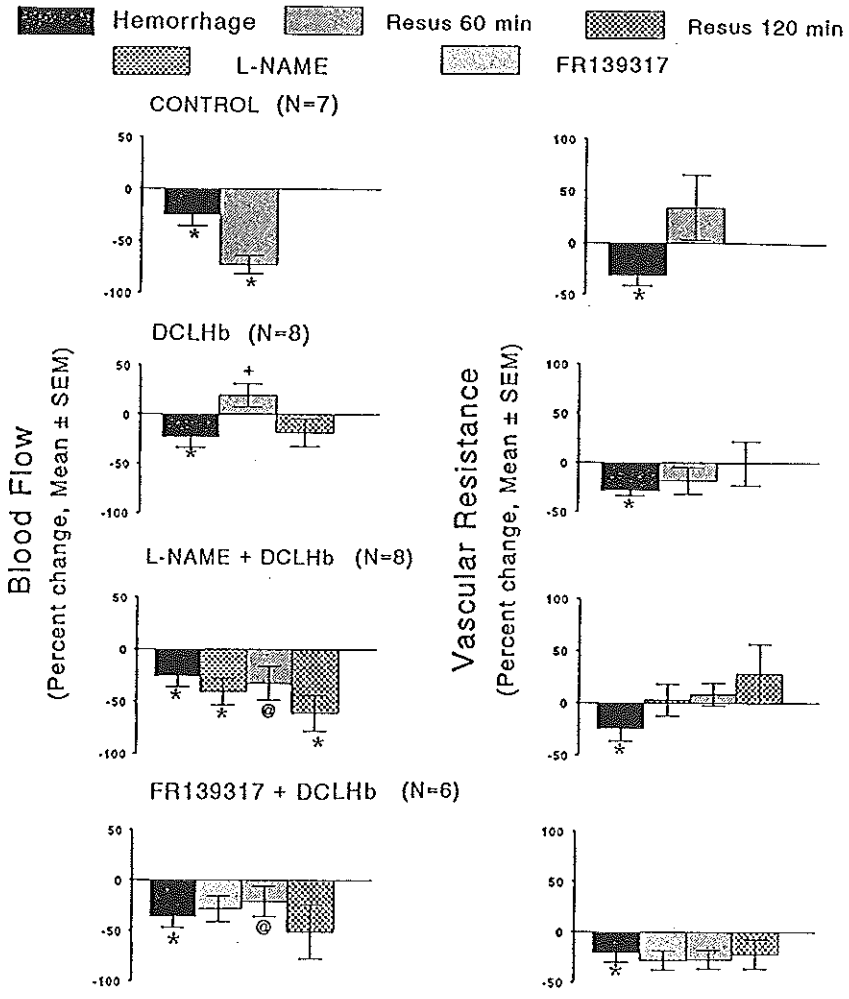

Fig. 3 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (4 mg/kg, i.v.) pretreated rats on percent change in blood flow and vascular resistance in the brain following hemorrhage (solid bars) and 60 min (hatched bars) and 120 min (cross hatched bars) after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

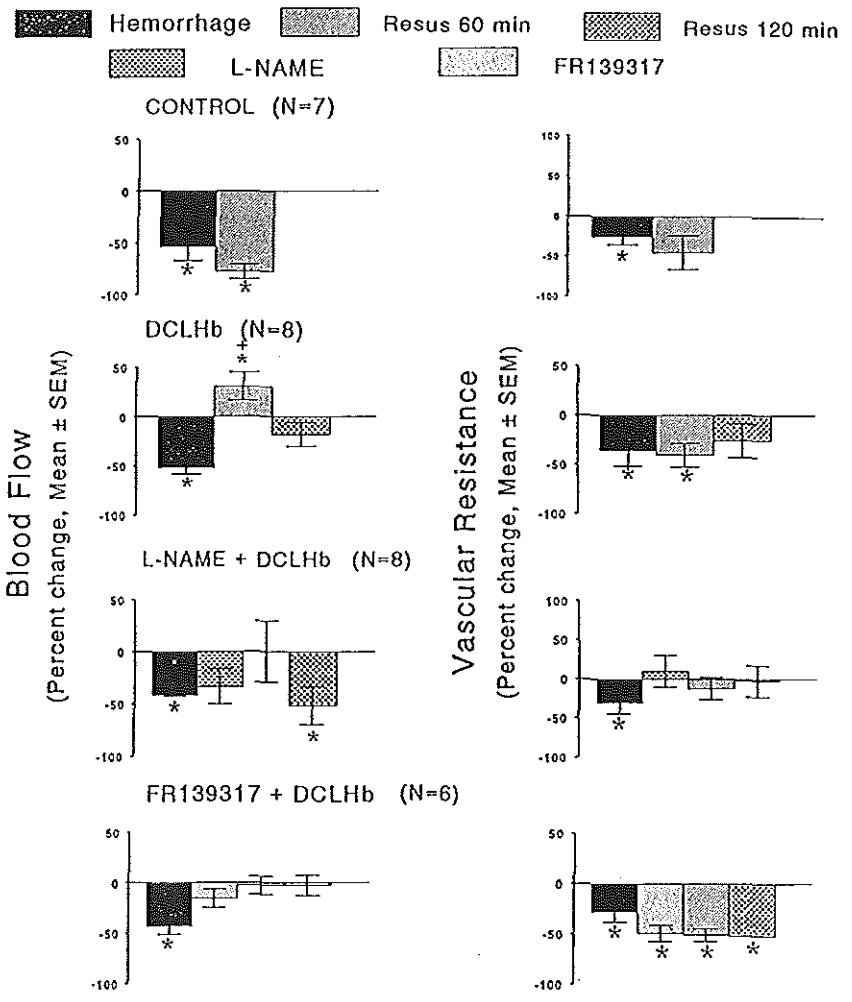

Fig. 4 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (4 mg/kg, i.v.) pretreated rats on percent change in blood flow and vascular resistance in the heart following hemorrhage (solid bars) and 60 min (hatched bars) and 120 min (cross hatched bars) after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

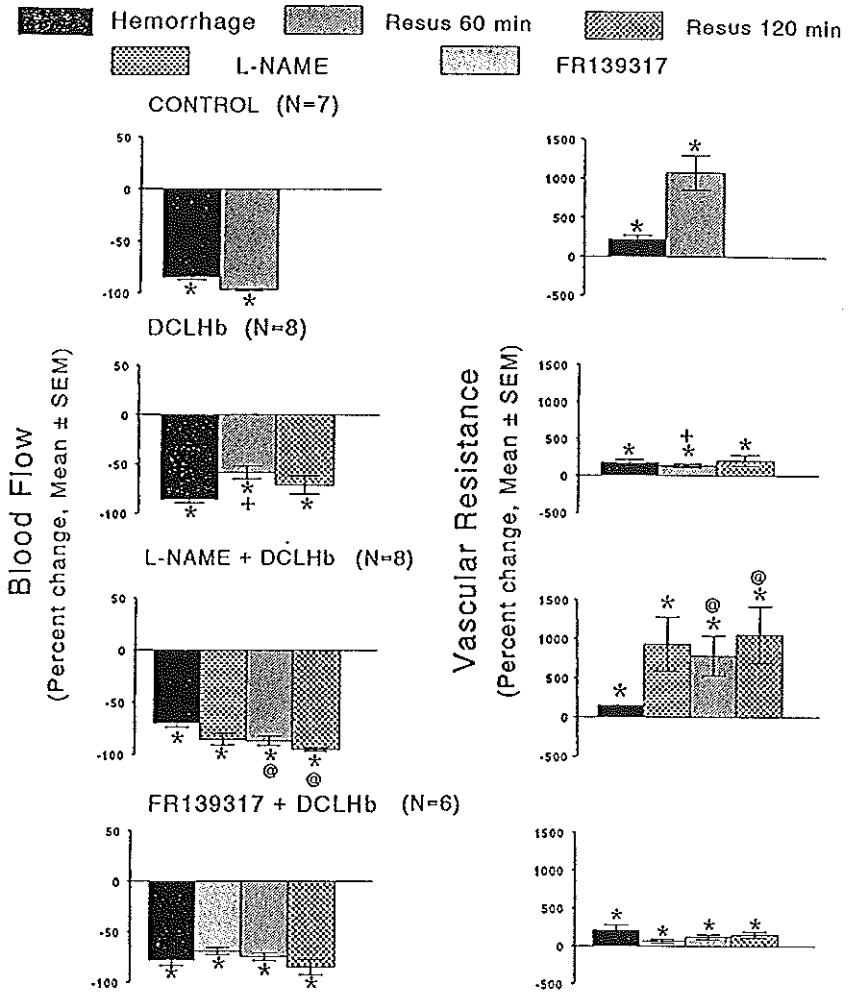

Fig. 5 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (4 mg/kg, i.v.) pretreated rats on percent change in blood flow and vascular resistance in the kidneys following hemorrhage (solid bars) and 60 min (hatched bars) and 120 min (cross hatched bars) after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

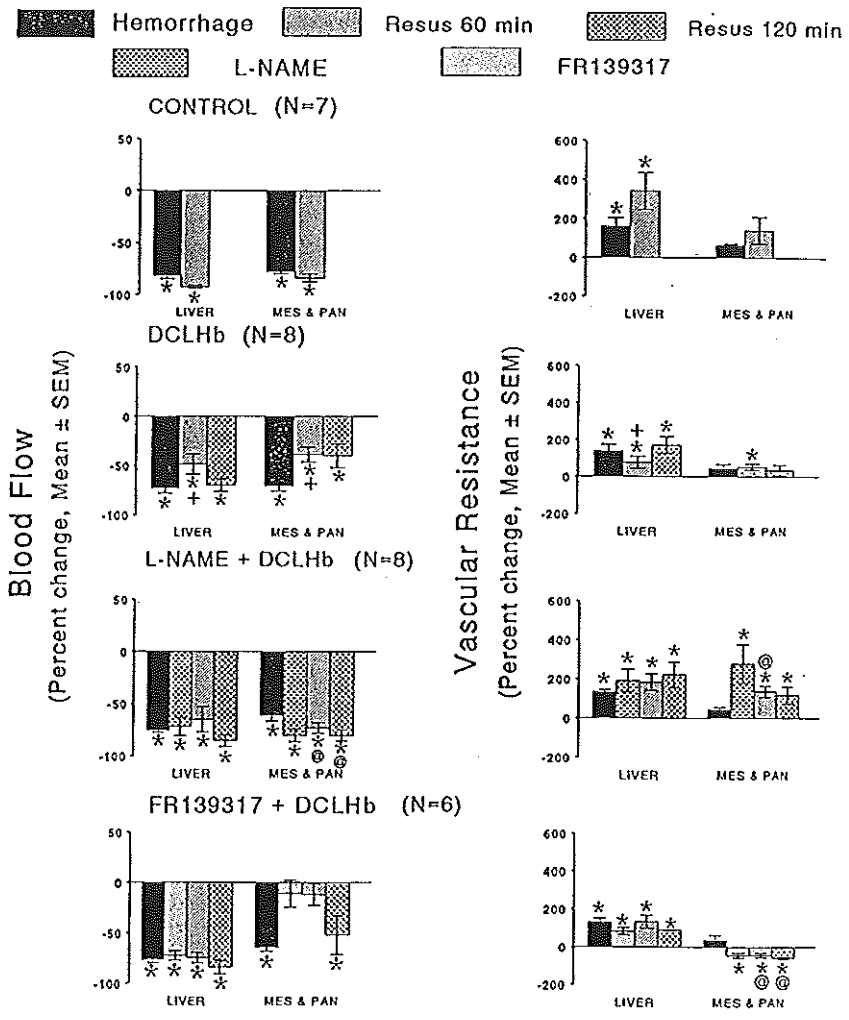

Fig. 6 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (4 mg/kg, i.v.) pretreated rats on percent change in blood flow and vascular resistance in the liver and mesentery & pancreas (MES & PAN) following hemorrhage (solid bars) and 60 min (hatched bars) and 120 min (cross hatched bars) after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

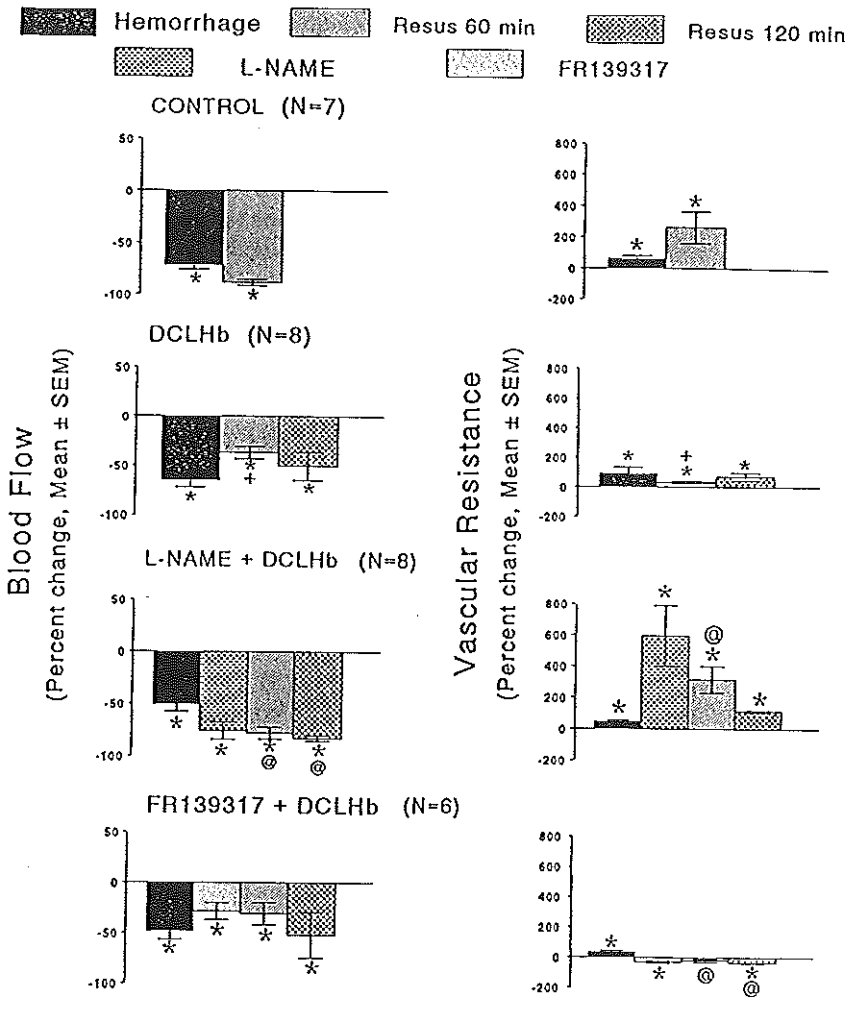

Fig. 7 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (4 mg/kg, i.v.) pretreated rats on percent change in blood flow and vascular resistance in the gastrointestinal tract following hemorrhage (solid bars) and 60 min (hatched bars) and 120 min (cross hatched bars) after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

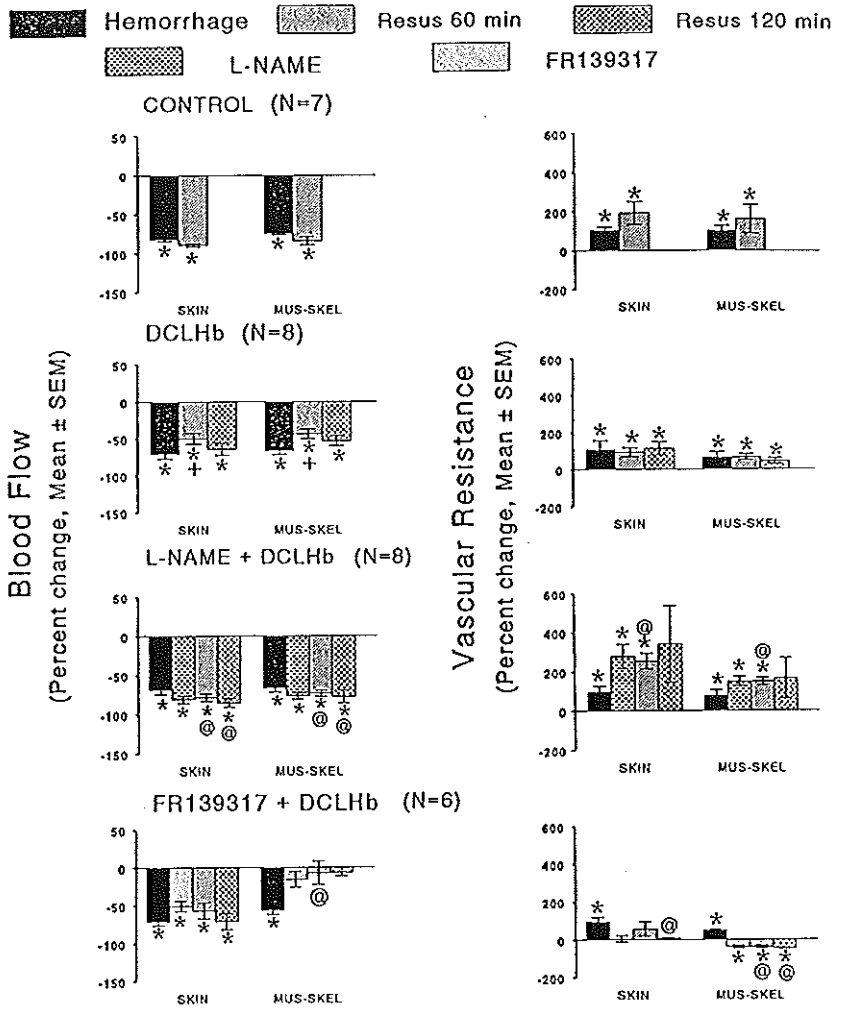

Fig. 8 The effect of vehicle (control, RL 4 ml/kg, i.v.), DCLHb (400 mg/kg, i.v.), DCLHb in L-NAME (10 mg/kg, i.v.) pretreated and DCLHb in FR139317 (4 mg/kg, i.v.) pretreated rats on percent change in blood flow and vascular resistance in the skin and musculoskeletal system (MUS-SKEL) following hemorrhage (solid bars) and 60 min (hatched bars) and 120 min (cross hatched bars) after resuscitation. *Indicates significant difference compared to baseline, #indicates significant difference compared to control group and @indicates significant difference compared to DCLHb treated group.

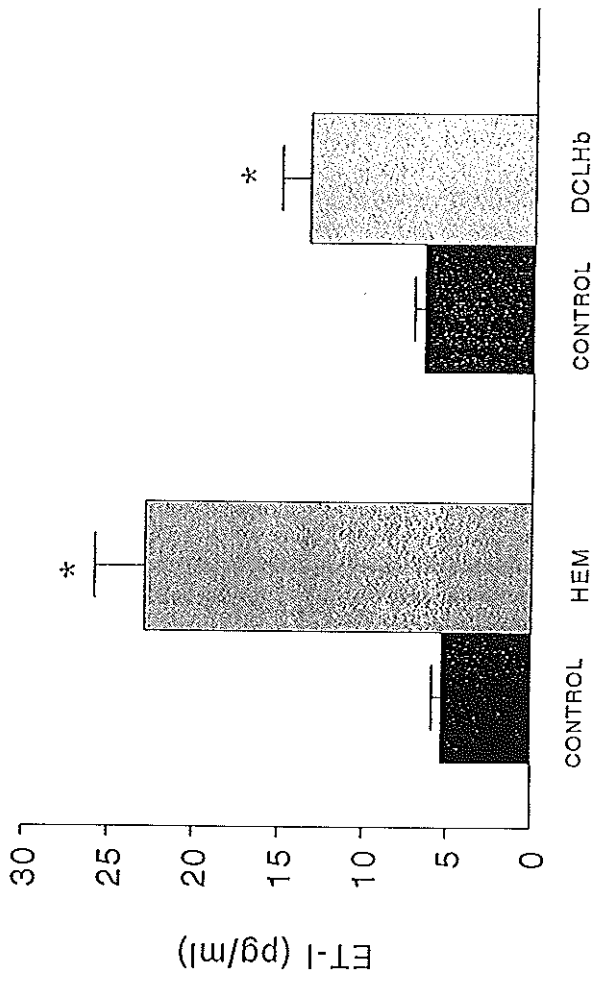

Fig. 9 Blood plasma concentration of ET-1 in control and hemorrhaged rats and in rats treated with vehicle (RL, 4 ml/kg, i.v.; control) or DCLHb (400 mg/kg, i.v.). *Indicates significant difference compared to control group.

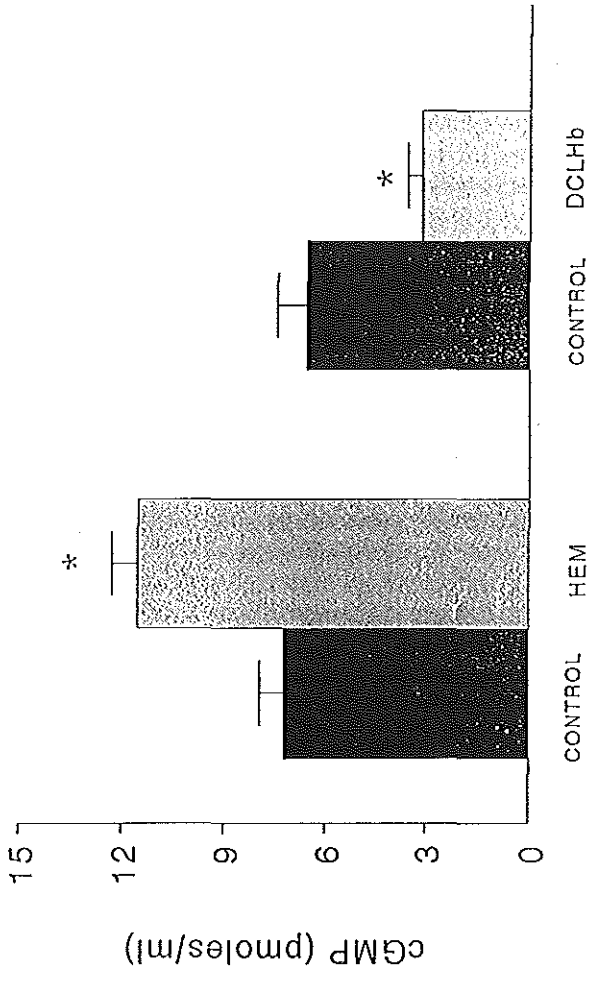

Fig. 10 Blood plasma concentration of cGMP in control and hemorrhaged rats and in rats treated with vehicle (RL, 4 ml/kg, i.v.; control) or DCLHb (400 mg/kg, i.v.). *Indicates significant difference compared to control group.

Effect on regional blood circulation

Hemorrhage decreased blood flow to the brain by >23% ($p < 0.01$) and vascular resistance in the brain by >21% ($p < 0.01$). Resuscitation with vehicle further decreased blood flow to the brain without altering the vascular resistance. On the other hand, resuscitation with DCLHb significantly increased ($p = 0.0001$) blood flow to the brain without altering vascular resistance when compared to vehicle resuscitated animals. Pretreatment with L-NAME attenuated ($p = 0.02$) the DCLHb induced increase in blood flow to the brain without altering vascular resistance. Pretreatment with FR139317 also attenuated ($p = 0.05$) the DCLHb induced increase in blood flow to the brain without altering vascular resistance (Fig. 3).

Hemorrhage decreased blood flow to the heart by >43% ($p < 0.001$) and vascular resistance in the heart by >26% ($p < 0.01$). Resuscitation with vehicle further decreased blood flow to the heart without altering vascular resistance. However, resuscitation with DCLHb increased ($p = 0.0001$) blood flow to the heart without altering vascular resistance when compared to vehicle resuscitated animals. In L-NAME or FR139317 pretreated rats, DCLHb did not produce an increase in blood flow to the heart. The vascular resistance in the heart was not affected in L-NAME treated rats but was decreased in FR139317 treated rats (Fig. 4).

Hemorrhage decreased blood flow to the kidneys by >70% ($p < 0.0001$) and increased vascular resistance in the kidneys by >149% ($p < 0.0001$). Resuscitation with vehicle further decreased blood flow and increased vascular resistance. However, resuscitation with DCLHb attenuated the hemorrhage induced decrease ($p = 0.0002$) in blood flow and increase ($p = 0.0006$) in vascular resistance as compared to vehicle resuscitated rats. Pretreatment with L-NAME significantly attenuated ($p = 0.004$) the effect of DCLHb on blood flow and vascular resistance in the kidneys. Pretreatment with FR139317 did not alter the effect of DCLHb on blood flow and vascular resistance in the kidneys (Fig. 5).

Hemorrhage significantly decreased blood flow to the liver by >73% ($p < 0.0001$) and to the mesentery and pancreas by >61% ($p < 0.0001$). Hemorrhage increased ($p < 0.0001$) vascular resistance in the liver by >132% but vascular resistance in the mesentery and pancreas was not significantly affected. Resuscitation with vehicle further decreased blood flow and increased vascular resistance in the liver and mesentery and pancreas. Rresuscitation with DCLHb attenuated ($p = 0.01$) the hemorrhage induced decrease in blood flow in both the liver and mesentery and pancreas when compared to vehicle resuscitated rats. Resuscitation with DCLHb attenuated ($p = 0.02$) the hemorrhage induced increase in vascular resistance in the liver without affecting vascular resistance in the mesentery and pancreas. Pretreatment with L-NAME or FR139317 did not alter the blood flow or vascular resistance in the liver. Pretreatment with L-NAME attenuated ($p = 0.02$) the effect of DCLHb on blood flow and vascular resistance in the mesentery and pancreas, while pretreatment with FR139317 attenuated ($p = 0.001$) vascular resistance in the mesentery and pancreas without affecting blood flow (Fig. 6).

Hemorrhage decreased blood flow to the GIT by > 48% ($p < 0.008$) and increased vascular resistance in the GIT by > 48% ($p < 0.008$). Resuscitation with vehicle further decreased blood flow and increased vascular resistance in the GIT. Resuscitation with DCLHb significantly attenuated the hemorrhage induced decrease ($p = 0.002$) in blood flow to the GIT when compared to vehicle resuscitated rats. Resuscitation with DCLHb significantly attenuated the hemorrhage induced increase ($p = 0.04$) in vascular resistance in the GIT. Pretreatment with L-NAME significantly attenuated the effect of DCLHb on blood flow ($p = 0.0002$) and vascular resistance ($p = 0.008$) in the GIT of hemorrhaged rats. Pretreatment with FR139317 significantly blocked ($p = 0.008$) the DCLHb induced changes in vascular resistance in the GIT of hemorrhaged rats (Fig.7).

Hemorrhage decreased blood flow in the skin by >62% ($p < 0.005$) and in the musculoskeletal system by >57% ($p < 0.005$). Hemorrhage increased ($p < 0.0001$) vascular resistance in the skin by >95% and in the musculoskeletal system by >51%. Resuscitation with vehicle further decreased blood flow and increased vascular resistance in both skin and musculoskeletal system. Resuscitation with DCLHb attenuated the hemorrhage induced decrease in blood flow to the skin ($p = 0.0003$) and musculoskeletal system ($p = 0.0005$), without altering the vascular resistance compared to vehicle resuscitated rats. Pretreatment with L-NAME attenuated the effect of DCLHb on blood flow and vascular resistance in the skin ($p = 0.005$) and musculoskeletal system ($p = 0.007$). Pretreatment with FR139317 attenuated ($p = 0.04$) the effect of DCLHb on blood flow and vascular resistance in the musculoskeletal system (Fig. 8).

Effect of hemorrhage and DCLHb on blood plasma ET-1 concentration

The basal blood plasma ET-1 concentration was 5.35 ± 0.55 pg/ml. However, following hemorrhage ET-1 concentration increased ($p = 0.0001$) to 22.9 ± 2.9 pg/ml. Administration of vehicle (Ringer's lactate; 4 ml/kg, i.v.) did not alter the plasma concentration of ET-1 (6.5 ± 0.61 pg/ml). However, administration of DCLHb produced a significant ($p = 0.003$) increase in ET-1 concentration (13.4 ± 1.7 pg/ml) (Fig. 9).

Effect of hemorrhage and DCLHb on blood plasma cGMP concentration

The basal blood plasma cGMP concentration was 7.25 ± 0.70 pmoles/ml. However, following hemorrhage cGMP concentration increased ($p = 0.001$) to 11.55 ± 0.74 pmoles/ml. Administration of vehicle (Ringer's lactate; 4 ml/kg, i.v.) did not alter the plasma concentration of cGMP (6.60 ± 0.86 pmoles/ml). However, administration of DCLHb produced a significant ($p = 0.003$) decrease in cGMP concentration (3.14 ± 0.42 pmoles/ml) (Fig. 10).

13.4 Discussion

Identification and control of bleeding and restoration of intravascular volume by resuscitation is the mainstay of initial therapy in the hemorrhaged patient. Successful recovery

is dependent upon rapid restoration of delivery, perfusion and subsequent utilization of oxygen for oxidative phosphorylation by the respiratory enzymes of the mitochondria to prevent tissue damage and multiple organ failure and improve survival (Moore *et al.*, 1992; Shoemaker *et al.*, 1988). Transfusion with homologous donor blood is still the conventional treatment for hemorrhagic shock (Rabinovici *et al.*, 1995). Unfortunately, there are several limitations to the use of blood such as, transmission of human immunodeficiency virus (Cumming *et al.*, 1989) and hepatitis (Alter *et al.*, 1989), storage and delivery problems and limited supply (Rabinovici *et al.*, 1995; Bunn, 1993). Though resuscitation with hypervolemic isotonic crystalloid solutions has been commonly used to treat hemorrhagic shock, there is a resurgence in the evaluation of its usefulness due to the high volumes necessary for resuscitation and poor survival rate (Vassar and Holcroft, 1992). Hypertonic solutions (7-7.5% NaCl) in small volumes, either alone or in combination with hyperoncotic colloids, have been effectively used in animal models of hemorrhage and in clinical trials (Frey *et al.*, 1994; Mazzoni *et al.*, 1994). The need exists for developing low volume resuscitative fluids which would overcome the limitations of homologous blood transfusion, and in addition, would possess excellent oxygen carrying capacity. DCLHb, a hemoglobin based blood substitute, has been found to be an effective resuscitative solution in animal models of hemorrhage (Przybelski *et al.*, 1991a; Malcolm *et al.*, 1992; Schultz *et al.*, 1993b).

In the present study a rodent model of fixed pressure hemorrhage without tissue trauma was used (Stephan *et al.*, 1987). This hemorrhagic model was made more severe by maintaining the hypotension for 90 min from the onset of hemorrhage to reach a base deficit of greater than -12 mmol/l (Schultz *et al.*, 1993b). In this irreversible situation, the compensatory mechanisms fail (decompensatory phase), and there is a tremendous decrease in blood flow to all organs, causing hypoperfusion leading to tissue hypoxia and end organ failure (Bond and Johnson, 1985). The present study shows that in control rats, vehicle was not effective in improving the time of survival, increasing oxygen consumption, or reversing base deficit. This suggests that persistent oxygen debt and inadequate resuscitation was achieved with vehicle. On the other hand, DCLHb increased the time of survival. DCLHb also attenuated the base deficit 60 min after resuscitation, though prehemorrhagic levels were not attained. The reversal of hemorrhage induced base deficits in the present study are comparable with another study in conscious rats using a 7% w/v solution of DCLHb at 50-100% of shed blood volume (Schultz *et al.*, 1993b). DCLHb significantly increased oxygen consumption and restored it to basal levels. Studies conducted to determine systemic hemodynamics showed that the vehicle was ineffective in improving the hemorrhage induced decrease in MAP and CO. On the other hand, resuscitation with DCLHb produced a significant pressor effect which was due to a significant increase in SV and CO. DCLHb significantly increased HR in hemorrhaged rats. Similar results have been observed in

other studies conducted in hemorrhaged rats using a 7-14% w/v solution of DCLHb at 50-100% of shed blood volume (Malcolm *et al.*, 1992; Przybelski *et al.*, 1991a).

Hemorrhage produced a significant decrease in blood flow to the brain, heart, kidneys, liver, mesentery and pancreas, GIT, skin and musculoskeletal system. Administration of DCLHb produced a significant improvement in blood flow to all tissues. Since DCLHb significantly decreased the mortality at 120 min, increased the oxygen consumption, attenuated the base deficit, and improved systemic hemodynamic parameters and blood flow to all tissues, it appears that DCLHb is effective in hemorrhagic shock. Resuscitation with DCLHb can restore oxygen delivery and increase tissue perfusion, and therefore could prevent ischemic tissue damage and multiple organ failure following severe hemorrhage.

Studies on the efficacy of DCLHb in hemorrhagic resuscitation have previously noted improvements in base deficit (Schultz *et al.*, 1994; Hess *et al.*, 1993), transcutaneous oxygen tension (Przybelski *et al.*, 1991a), subcutaneous oxygen tension (Powell *et al.*, 1995), cardiac and renal functions (Hess *et al.*, 1989), and CO, SV and plasma lactate levels (Hess *et al.*, 1993). To our knowledge, no study has been performed to determine the mechanism involved in the efficacy of DCLHb as a resuscitative solution. DCLHb produces marked changes in systemic hemodynamics and regional blood circulation (Sharma and Gulati, 1994) through the adrenergic (Gulati and Rebello, 1994; Gulati and Sharma, 1994; Sharma and Gulati, 1995), ET (Gulati *et al.*, 1996; Schultz *et al.*, 1993a) and NO (Schultz *et al.*, 1993a; Sharma *et al.*, 1995; Katsuyama *et al.*, 1994) systems. It has been shown that DCLHb increases the plasma concentration of ET-1 (Gulati *et al.*, 1996) and is effective in attenuating the vasodilator effect of NO (Schultz *et al.*, 1993a; Sharma *et al.*, 1995) when administered to normal rats. It is not known whether an increase in ET-1 or removal of NO or both are responsible for the efficacy of DCLHb following hemorrhage. We have therefore determined the role of ET and NO systems in the modulation of cardiovascular effects of DCLHb following severe hemorrhage in rats.

NO is bound by hemoglobin and may be converted to nitrate through interaction with the heme group (Wennmalm *et al.*, 1992). NO is released in several pathological situations (Gibaldi, 1993). The NO system is often found to be involved in the regulation of regional blood circulation in normal animals. L-NAME, a NO synthase (NOS) inhibitor, blocked the increases in rat cortical blood flow due to stimulation of cerebrovascular parasympathetic system (Morita-Tsuzuki *et al.*, 1993) and decreased cerebral blood flow in goats (Fernandez *et al.*, 1993). Both L-NAME and another NOS inhibitor, N^G-Nitro-L-arginine, produced constriction of coronary arterioles in dogs (Jones *et al.*, 1993). L-NAME decreased renal blood flow and increased renal vascular resistance in species ?? (Sigmon *et al.*, 1993), attenuated the vasodilation of muscular arterioles in response to acetylcholine in hamsters (Hester *et al.*, 1993), and decreased cerebral blood flow and increased cerebral vascular resistance in dogs (Saito *et al.*, 1994). Severe hemorrhagic hypotension causes hyporeactivity of the cardiovascular system to

catecholamines which is mediated by an increase in NOS activity and release of NO (Thiemermann *et al.*, 1993). Most of the studies using NOS inhibitors have shown a beneficial role of NO in hemorrhage. Resuscitation with L-NAME (10 mg/kg, i.v.) has been found to increase MAP in urethane anaesthetized rats hemorrhaged for 20 min (Zingarelli *et al.*, 1992). In another study, L-NAME (0.3-30 mg/kg, i.v.) increased MAP in urethane anaesthetized hemorrhaged rats in a dose-dependent manner (Chyu *et al.*, 1992). L-NAME (44 mg/kg, i.v.) pretreatment increased MAP but decreased HR in conscious hemorrhaged rabbits (Koch *et al.*, 1995). Another NOS inhibitor N^G-methyl-L-arginine (30 mg/kg, i.v.) has been shown to restore MAP in hemorrhaged rats due to an increase in systemic vascular resistance (Klabunde *et al.*, 1993), while infusion of N^G-monomethyl-L-arginine (1.2 mg/kg/min) increased MAP in hemorrhaged rats partly due to an increase in renal vascular resistance (Lieberthal *et al.*, 1991).

Endogenous NO mediates most of its actions through cGMP (Thiemermann, 1994), and the hyporeactivity to vasoconstrictors following hemorrhage could be due to an increased production of NO and cGMP as a result of enhanced activity of NOS (Thiemermann *et al.*, 1993). It appears that an increase in cGMP levels following severe hemorrhage will lead to loss of vascular tone and hyporeactivity of blood vessels to vasoconstrictors and is deleterious to the survival of the animal. The present study clearly shows that cGMP concentration is increased following hemorrhage, while DCLHb produced a significant decrease in cGMP concentration in the blood plasma. It is possible that following hemorrhage an increase in the release of NO occurs leading to an increase in cGMP concentration in the blood plasma. DCLHb when administered would remove NO and thus decrease the concentration of cGMP, as observed in the present study. The removal of NO and decrease in cGMP is likely to contribute toward restoring the vascular tone and responsiveness. To test this hypothesis DCLHb was administered in rats pretreated with L-NAME. In rats pretreated with L-NAME the DCLHb induced increase in base deficit and oxygen consumption was attenuated. Pretreatment with L-NAME also attenuated the DCLHb induced increase in MAP and improvement in blood flow to the brain, GIT, kidneys, mesentery and pancreas, skin, and musculoskeletal system. If removal of increased NO formed following hemorrhage is responsible for the therapeutic effect of DCLHb, L-NAME should have potentiated the beneficial effects of DCLHb in hemorrhagic situations. In contrast, L-NAME attenuated the beneficial effects of DCLHb.

The concentration of blood plasma ET-1 was found to be significantly increased in rats following hemorrhage. The elevation of plasma ET-1 concentration has been reported following hemorrhage in rats (Vemulapalli *et al.*, 1994; Zimmerman *et al.*, 1994) and dogs (Chang *et al.*, 1993). In normal rats the circulating ET-1 level is low and ET-1 is preferentially released toward abluminal side of the endothelial cells. Hemorrhage leads to an increase in plasma ET-1 concentration either due to an increase in the synthesis or release of ET-1 as a consequence of hemodynamic changes. The elevation of ET-1 level could also be due to the activation of stress

hormones, the coagulation cascade, or as a result of a decrease of blood flow to the kidneys and lungs causing decreased clearance of ET-1. In the present study, hemorrhage produced a significant increase in plasma ET-1 levels. DCLHb also significantly increased plasma ET-1 levels, though this increase was significantly less than the hemorrhage induced increase in ET-1 levels. This is in agreement with another report in which oxyhemoglobin solution was found to increase ET-1 concentration in cultured bovine pulmonary artery endothelial cells and augmented ET production following platelet-mediated stimulation of ET-1 production (Ohlstein and Storer, 1992). The DCLHb induced increase in MAP, TPR and blood flow to the brain and musculoskeletal system was significantly attenuated in FR139317 pretreated rats. Pretreatment with FR139317 significantly attenuated the DCLHb induced decrease in base deficit, and increase in oxygen consumption and survival time. It is therefore possible that an increase in ET-1 concentration in the blood plasma following hemorrhage is a part of compensatory response and FR139317, an ET receptor antagonist, attenuated the beneficial effects of DCLHb. Interestingly, neither L-NAME nor FR139317 altered the DCLHb induced improvement in blood flow to the heart. It was recently shown that in normal rats ET receptors in the heart are different from those found in other vascular beds (Gulati *et al.*, 1995a). It may be possible that under hemorrhagic conditions this distinction is maintained.

DCLHb has also been found to increase the ET-1 concentration (Gulati *et al.*, 1996; Gulati *et al.*, 1995b) and scavenge NO (Sharma *et al.*, 1995) in normal rats. Therefore, restoration of arteriolar tone could be achieved as a result of removal of NO and an increase in the concentration of ET by DCLHb. In summary, hemorrhage produced a significant increase in plasma concentration of cGMP, but L-NAME pretreatment attenuated the beneficial effects of DCLHb in hemorrhaged rats. On the other hand, hemorrhage produced a significant increase in plasma ET-1 concentration and FR139317 (an ET_A receptor antagonist) attenuated the beneficial effects of DCLHb in hemorrhaged rats. The increase in plasma ET-1 concentration following hemorrhage appears to be a part of the normal compensatory response. Since inhibition NO formation by L-NAME decreased the efficacy of DCLHb, therefore NO removal by DCLHb may not be contributing towards the efficacy of DCLHb in hemorrhaged rats. However, ET antagonist decreased the efficacy of DCLHb, therefore an increase in plasma ET-1 concentration by DCLHb may be contributing towards the efficacy of DCLHb in hemorrhaged rats. It is concluded that ET mechanism is more important in the beneficial effects of DCLHb in hemorrhaged rats than NO mechanism.

13.5 Acknowledgment

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Part 7

General Discussion

Chapter 14

General discussion, and implications for future research

14.1 General discussion

The following are the major findings as a results of the work presented in this thesis:

1. The mechanisms involved in the cardiovascular actions of DCLHb have been described for the first time in normal and hemorrhage rats. It has been shown that purification and chemcial modification of hemoglobin alters its cardiovascular actions. Although adrenergic mechanisms are invovled in the cardiovascular actions of hemoglobin solutions but the effect of DCLHb on regional blood circulation are extremely different from that produced by equipressor doses of norepinephrine.

2. The results of this thesis clearly indicate that DCLHb is not a simple oxygen carrying solution but has pharmacological properties. The efficacy of DCLHb in hemorrhaged situation is due to its oxygen carrying abilitites and more importantly due to its ability to produce significant effect on the cardiovascular system.

3. The role of NO and ET in hemorrhagic shock has been elucidated as a result of the present studies. It has been shown that removal of NO is important in the treatment of hemorrhagic shock.

4. Present study also demonstrates for the first time that in hemorrhagic shock ET could an important mediator which could be used clinically in the management of hemorrhagic shock.

5. This is the first study which establishes the mechanism of action of DCLHb, a hemoglobin based therapeutic agent.

Diaspirin cross-linked hemoglobin (DCLHb^{TM}) (Baxter Healthcare Corporation) is a promising resuscitative fluid. The effect of DCLHb (400 mg/kg, iv), on regional circulation and systemic hemodynamics was studied in male Sprague-Dawley rats using a radioactive microsphere technique. Infusion of an equal volume of saline did not produce any significant change in systemic hemodynamics or regional circulation. DCLHb produced an increase in the mean blood pressure which lasted for more than 60 min. Heart rate, cardiac output and stroke volume were not significantly affected, while total peripheral resistance was increased after the administration of DCLHb. DCLHb produced significant increases in blood flow to the heart, gastrointestinal tract (GIT), portal system and skin. The blood flow to kidney, brain and

musculoskeletal system was not significantly affected by DCLHb. The vascular resistance was not altered in the heart, brain, GIT, portal system, kidney or skin, but there was a marked increase in the vascular resistance in the musculoskeletal system. There was a significant increase in the percentage of cardiac output to visceral organs like heart, GIT and portal system, while a marked decrease in the percent cardiac output to musculoskeletal system was observed with DCLHb (Chapter 2). It is concluded that DCLHb produces a redistribution of cardiac output from the musculoskeletal system to the vital organs.

The effect of DCLHb was compared with unmodified stroma-free hemoglobin (SFHb). SFHb and DCLHb increased mean arterial blood pressure without affecting heart rate. SFHb produced a 24.9 % decrease in the cardiac output while DCLHb produced an 44.8 % increase in the cardiac output. Stroke volume was decreased (-27.3 %) by SFHb and increased (+36.4 %) by DCLHb. Total peripheral resistance increased with both SFHb and DCLHb. DCLHb increased blood flow to the heart, spleen, stomach, small intestine and skin, and had no effect on blood flow to the brain, kidneys, liver, mesentery, pancreas, caecum, large intestine and musculo-skeletal system. In contrast, in animals infused with SFHb, blood flow decreased to the kidneys, liver and spleen, increased to the heart, small intestine and skin, and had no effect to the brain, caecum, large intestine and musculo-skeletal system. DCLHb had no effect on vascular resistance in any organ except for an increase in the musculo-skeletal system. In contrast, SFHb increased vascular resistance in the kidneys, liver, spleen, skin, mesentery and pancreas, and had no effect on vascular resistance in the musculo-skeletal system, brain, heart, stomach, small intestine, caecum and large intestine. SFHb had no effect on distribution of cardiac output to the brain, gastrointestinal tract (GIT), kidneys, skin, musculo-skeletal and portal system, while DCLHb significantly decreased the percent cardiac output to the musculo-skeletal system. DCLHb did not affect the distribution of cardiac output to the brain, GIT, kidneys, skin and portal system. SFHb and DCLHb increased the percent cardiac output to the heart (Chapter 3). It is concluded that similar concentrations and doses of DCLHb and SFHb produce different effects on the regional blood circulation and systemic hemodynamics.

Studies were conducted to compare the cardiovascular effects of DCLHb with that of norepinephrine (NE) at equipressor doses. DCLHb (400 mg/kg, i.v.) produced a pressor effect which was equal to that produced by norepinephrine (NE) (25 μg/kg/min i.v. infusion). Total peripheral resistance was increased by DCLHb and more significantly by NE. Heart rate was not affected by DCLHb but was significantly increased by NE. The cardiac output and stroke volume were insignificantly increased by DCLHb but were significantly decreased by NE. DCLHb and NE produced a significant increase in blood flow to the heart. The vascular resistance in the heart was not affected by DCLHb but was decreased by NE. DCLHb did not

affect the renal and brain circulation but NE in kidneys decreased the blood flow and increased the vascular resistance, while in brain increased the blood flow and decreased the vascular resistance. DCLHb increased the blood flow to the stomach and small intestine. The vascular resistance was not affected by DCLHb in the gastrointestinal tract. NE did not affect the blood circulation in the gastrointestinal tract. The blood flow to spleen was increased by DCLHb and there was no change in the vascular resistance. NE insignificantly decreased the blood flow to spleen and significantly increased the vascular resistance. The blood circulation to the mesentry and pancreas was not affected by DCLHb, while NE increased the blood flow without affecting the vascular resistance. DCLHb produced a significant increase in the blood flow to the skin without affecting the vascular resistance, while NE did not affect the blood flow but increased the vascular resistance. DCLHb did not affect the blood flow to musculo-skeletal system but increased the vascular resistance, while NE decreased the blood flow and increased the vascular resistance (Chapter 4). In summary, although the pressor effect of DCLHb and NE at the doses studied is equal, DCLHb did not decrease the blood flow to any organ, whereas NE produced significant decreases in blood flow to several organs. It is concluded that the blood flow to most of the organs is either increased or not affected by DCLHb.

To determine the role of the central nervous system (CNS) and the peripheral vascular system in the pressor effect of DCLHb, experiments were performed in cervical sectioned rats. Intravenous administration of DCLHb produced an increase in blood pressure in cervical sectioned animals, which was similar to that observed in normal rats. To test whether the pressor effect was due to release of catecholamines or other pressor substances from the adrenal medulla, DCLHb was administered to bilateral adrenal demedullated rats. It was found that DCLHb produced a pressor effect in bilateral adrenal demedullated rats that was similar to normal rats. To determine whether DCLHb alters the responsiveness of peripheral vascular adrenoceptors, the effect of DCLHb pretreatment on the blood pressure and heart rate responses of adrenergic agonists was studied. DCLHb significantly potentiated (66.7%) the pressor response to norepinephrine (0.5 to 2.0 μg/kg, i.v.) but did not affect the heart rate response to norepinephrine. Phenoxybenzamine completely blocked the DCLHb induced potentiation of the norepinephrine responses. Phenylephrine produced a dose dependent (5 to 20 μg/kg, i.v.) pressor and reflex bradycardic effect. DCLHb significantly potentiated the pressor (40.6%) and bradycardic (-22.8%) effect of phenylephrine which were completely blocked by prazosin. Clonidine produces a fall in blood pressure by acting on the central α-adrenoceptors, and a rise in blood pressure by stimulating the peripheral vascular α-adrenoceptors. DCLHb produced a marked potentiation of the pressor response to clonidine (75 μg/kg, i.v.) that masked the central depressor effect. Prazosin pretreatment did not attenuate the DCLHb induced potentiation of the pressor effect of clonidine in intact rats. However, yohimbine pretreatment completely blocked the

DCLHb induced potentiation of the clonidine induced pressor response in intact rats. In order to exclude the contribution of a centrally induced cardiovascular effect of clonidine, further studies were carried out in cervical sectioned rats. DCLHb markedly potentiated the pressor effect of clonidine (25 μg/kg, i.v.) in cervical sectioned rats. This potentiation could be attenuated by prazosin and yohimbine. Pretreatment with either yohimbine (2 mg/kg, i.v.) or prazosin (1 mg/kg, i.v.) significantly attenuated the DCLHb induced pressor effect. The attenuation of DCLHb induced pressor effect was more marked with prazosin as compared to yohimbine (Chapter 5). It is concluded that the pressor effect of DCLHb is not mediated through the CNS, however, it appears that in rats both α_1- and α_2-adrenoceptors in the peripheral vascular system are sensitized by DCLHb.

Studies were conducted to determine the effect of adrnergic receptor antagonists on the cardiovascular effect of DCLHb. It was found that infusion of DCLHb in prazosin (1 mg/kg, i.v.) treated rats did not show any significant pressor effect, but reversed the hypotensive effect of prazosin. Cardiac output and stroke volume were significantly increased and total peripheral resistance decreased in prazosin treated rats as compared to control (untreated) rats. DCLHb significantly increased blood flow to the heart, gastrointestinal tract, portal system (spleen), and skin of control rats. Blood flow to the brain, kidneys, and musculo-skeletal system was not altered following the infusion of DCLHb in control rats. Infusion of DCLHb in prazosin treated rats produced a significant increase in blood flow to the brain, heart, kidneys, gastrointestinal tract, portal system, skin and musculoskeletal system. In summary, prazosin pretreatment blocked the pressor effect of DCLHb, however, blood flow to the heart, brain, gastrointestinal tract, portal system, kidneys, skin and musculoskeletal system was increased by DCLHb (Chapter 6). In yohimbine (2 mg/kg, iv) pretreated animals, DCLHb did not produce any change in heart rate, stroke volume, cardiac output and total peripheral resistance, but a slight increase in blood pressure was observed compared to baseline values obtained after the administration of yohimbine. The increase in blood pressure induced by DCLHb was significantly blocked by pretreatment with yohimbine. Yohimbine (2 mg/kg, iv) per se decreased blood pressure, while other systemic hemodynamics parameters were not affected. In yohimbine pretreated animals,DCLHb produced an increase in blood flow to the heart, brain (cerebellum and brain stem), liver, small intestine, caecum, spleen, mesentery and pancreas, kidneys, skin and musculoskeletal system, while blood flow to the stomach and large intestine was not affected. Yohimbine pretreatment significantly attenuated the DCLHb induced increase in blood flow to the large intestine, mesentery and pancreas (Chapter 7). Adrenergic antagonists may be useful in attenuating the pressor effect of DCLHb while maintaining the regional perfusion.

The role of nitric oxide (NO) in the cardiovascular actions of diaspirin crosslinked hemoglobin (DCLHb) was studied in anesthetized rats. The regional circulatory and systemic hemodynamic effects of DCLHb (400 mg/kg iv) were studied using a radioactive microsphere technique in control (untreated) and L-arginine (NO precursor) pretreated rats. DCLHb produced a significant increase in blood pressure, cardiac output, stroke volume and total peripheral resistance without affecting heart rate, when administered to control rats. L-arginine pretreatment significantly attenuated DCLHb-induced systemic hemodynamic effects. DCLHb induced increase in blood flow to the skin and spleen was completely and to the heart was partially blocked by L-arginine pretreatment suggesting that cardiovascular actions induced by DCLHb could be antagonized by NO precursor, L-arginine. NO synthase (NOS) inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester), produced significant increases in regional vascular resistance leading to a decrease in blood flow to all the organs excepting heart, where an increase in blood flow and decrease in vascular resistance was observed. DCLHb when administered in L-NAME pretreated rats, accentuated the decrease in blood flow to the GIT, spleen, mesentery and pancreas, skin and musculoskeletal system. These studies provide evidence that NO precursor L-arginine can attenuate the effects of DCLHb, and DCLHb can potentiate the effect of NOS inhibitor, L-NAME. The role of NO in the mechanism of action of DCLHb was further studied by estimating plasma cGMP in control, DCLHb treated, L-NAME treated and L-NAME followed by DCLHb treated rats. DCLHb and L-NAME significantly decreased the concentration of circulating cGMP in the blood plasma. L-NAME pretreatment potentiated DCLHb-induced decrease in cGMP levels. Since the formation of cGMP is stimulated by NO, these studies provide additional evidence for the involvement of NO in the mechanism of action of DCLHb (Chapter 8). It is concluded that NO plays an important role in the cardiovascular effects of DCLHb.

The role of endothelin (ET) in the cardiovascular actions of diaspirin crosslinked hemoglobin (DCLHb) was also determined. Infusion of DCLHb or SFHb increased mean arterial blood pressure and ET-1 levels in the blood plasma. The increase in plasma ET-1 concentration was significantly more marked with SFHb as compared to DCLHb treated rats. The concentration of ET-1 in the heart and brain regions was not altered in DCLHb and SFHb treated rats as compared to control. However, ET-1 concentration was significantly increased in the thoracic aorta and renal medulla of DCLHb treated rats. SFHb treated rats also showed a significant increase in ET-1 concentration in the thoracic aorta and renal medulla. The effect of SFHb on the renal medulla was found to be significantly greater than that of DCLHb (Chapter 9). Further studies were carried out to determine whether the cardiovascular effects of DCLHb are due to an increase in the conversion of proET-1 to ET-1 by endothelin converting enzyme (ECE). Phosphoramidon (4 mg/kg, i.v.) pretreatment attenuated the increase in mean arterial pressure and total peripheral

resistance, increase in blood flow to the heart, brain, kidneys, portal system and GIT, and the increase in vascular resistance in the mesentery and pancreas and musculoskeletal system induced by proET-1. However, in phosphoramidon treated rats, DCLHb increased mean arterial pressure, heart rate, cardiac output, and total peripheral resistance. DCLHb increased blood flow to the heart, brain, kidneys, GIT, portal system and skin when administered to phosphoramidon treated rats (Chapter 10). It is concluded that DCLHb induced systemic and regional circulatory effects could not be blocked by phosphoramidon, indicating that the cardiovascular effects of DCLHb are not due to an increase in the conversion of proET-1 to ET-1. The effect of BQ123, an ET receptor antagonist, on the cardiovascular actions of DCLHb was determined. The increase in blood pressure, cardiac output, stroke volume and total peripheral resistance by DCLHb was significantly blocked in BQ-123 (5 mg/kg/h, i.v.) treated rats as compared to control rats. The increase in blood flow to the heart, spleen and skin by DCLHb was significantly blocked in BQ-123 treated rats as compared to control rats. DCLHb produced an increase in the blood flow to the brain and a decrease in blood flow to the kidney and musculoskeletal system of BQ-123 treated rats as compared to control rats (Chapter 11). It is concluded that ET mechanism is invovled in the cardiovascular actions of DCLHb.

We have studied the cardiovascular effects of DCLHb (10% w/v solution, doses of 20%, 50%, and 100% i.v. of shed blood volume, SBV) and Ringer's lactate (20% of SBV as vehicle and 300% of SBV as resuscitative solution) in hemorrhaged rats using a radioactive microsphere technique. Hemorrhage was induced in anaesthetized male Sprague Dawley rats by bleeding them at a rate of approximately 0.5 to 1 ml/min until a mean arterial pressure (MAP) of 35-40 mmHg was achieved. This was maintained for up to 90 min from the onset of hemorrhage to reach a base deficit of less than -12 mmol/l. Hemorrhage significantly decreased MAP, cardiac output (CO), and stroke volume (SV), and increased total peripheral resistance (TPR). Hemorrhage significantly decreased regional blood flow to all the tissues and whole body oxygen consumption. Control rats were administered RL (20% of SBV, i.v.). This volume of RL (vehicle) did not produce any improvements in systemic hemodynamics, regional blood flow or oxygen consumption. DCLHb (20%, 50%, and 100% of SBV) produced significant improvements in systemic hemodynamics and regional blood flow. DCLHb at 20% of SBV improved the oxygen consumption and base deficit from 3.61 ± 0.34 to 4.73 ± 0.47 ml/min and -13.75 ± 0.79 to -10.97 ± 1.28 mmol/l, respectively. At 50% of SBV, DCLHb improved the oxygen consumption and base deficit from 3.49 ± 0.37 to 5.40 ± 0.60 ml/min and -13.28 ± 0.84 to -8.30 ± 0.89 mmol/l, respectively. At 100% of SBV, DCLHb improved the oxygen consumption and base deficit from 2.62 ± 0.37 to 5.69 ± 0.80 ml/min and -14.02 ± 2.13 to -7.50 ± 1.69 mmol/l, respectively. The mean survival time for RL (20% of SBV, i.v.) treated hemorrhaged rats was 63 min, DCLHb significantly increased the survival time (152 min in 20%, 221 min in 50% and 214 min in 100% of SBV) of hemorrhaged rats. RL (300% of SBV) when

administered at the rate of 1 ml/min for about 40 min, also produced significant improvement in base deficit, oxygen consumption, systemic hemodynamics and regional circulatory parameters (Chapter 12). It is concluded that DCLHb in a dose dependent manner produced a significant improvement in survival time, oxygen consumption, systemic hemodynamics, and regional blood circulation of hemorrhaged rats.

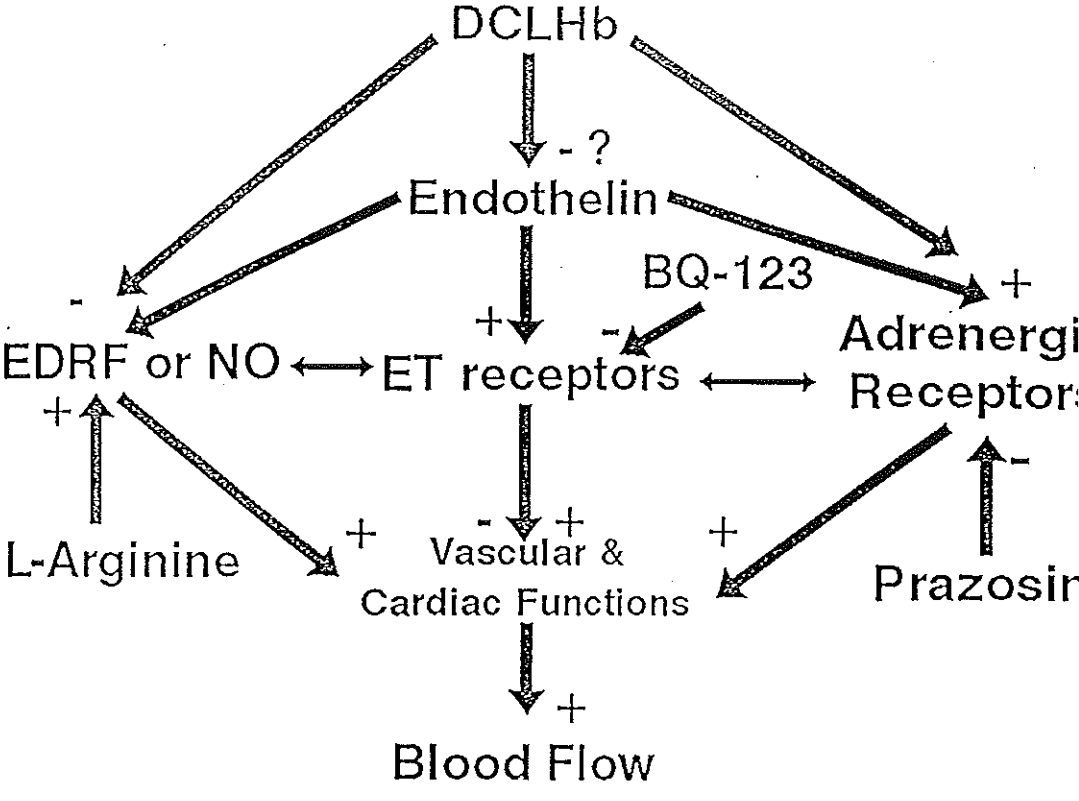

Fig. 1 Shows the mechanism of action of DCLHb. It has been found that treatment with L-arginine can block the systemic and regional circulatory effects of DCLHb; DCLHb did not produce any alterations in cardiovascular parameters in L-NAME pretreated rats; and DCLHb decreased plasma cGMP levels indicating that NO mechanism is involved in the action of DCLHb. Administration of DCLHb produces an increase in plasma ET-1 levels and the systemic and regional circulatory effects of DCLHb were blocked by pretreatment with BQ123 an ET receptor antagonist providing evidence for the involvement of ET system. Systemic and regional circulatory effects of DCLHb could also be blocked by pretreatment with adrenergic receptor antagonists like prazosin or yohimbine. It is now known that ET can regulate both NO and adrenergic systems.

Further studies were carried out in hemorrhage rats to determine the efficacy of DCLHb in hemorrhaged animals. Perfusion, concentration of moving red blood cells (CMBC) and red blood cell velocity were determined by using a laser Doppler flowmeter (Perimed, Model Periflux 4001), which utilizes the Doppler shift formed by a laser beam against the red blood cells. The animals were mechanically ventilated at a constant rate. Hemorrhage was induced by bleeding the rats at a rate of 0.5 to 1 ml/min till a mean arterial pressure of 35-40 mmHg was achieved. This was maintained for up to 30 min to reach a base deficit of less than -12 mmol/l. The arterial pH, pO_2, pCO_2 and THb were monitored. Hemorrhage significantly decreased blood pressure (-53.2 ± 2.9%) and arterial blood pH, pCO_2, THb, and increased pO_2. A significant decrease in the brain perfusion (-20.2 ± 3.27%) was observed following hemorrhage. The decrease in perfusion was due to a decrease in CMBC (-8.2 ± 2.8%) and red cell velocity (-5.1 ± 6.1%) in the brain. Hemorrhage produced a marked decrease (-73.6 ± 3.39%) in the renal perfusion, accompanied with a decrease in CMBC (-35.5 ± 8.9%) and red cell velocity (-57.2 ± 2.3%). Resuscitation with the vehicle (Ringer's lactate 4ml/kg, i.v.) did not produce any improvement in blood pressure, arterial blood pH, pO_2, pCO_2 and THb. A further decrease in the brain perfusion (-27.3 ± 2.6%) was observed 30 min following resuscitation with the vehicle. Although an increase in CMBC (10.3 ± 0.3%) was observed 30 min following resuscitation with the vehicle, a marked decrease in red cell velocity (-32.7 ± 2.5%) was observed in the brain. Renal perfusion was not altered by vehicle. Resuscitation with DCLHb (400 mg/kg, i.v.) produced an improvement in blood pressure, arterial blood pH, pO_2, pCO_2, and perfusion to the brain (9.0 ± 0.3%) and kidney (36.5 ± 10.7%). CMBC was not altered by DCLHb in the brain (-1.7 ± 4.1%) or kidney (11.3 ± 11.1%) but an increase in red cell velocity was observed in the brain (5.0 ± 4.2%) and kidney (23.2 ± 2.7%) following resuscitation with DCLHb. It is concluded that following hemorrhage it is the decrease in CMBC and red cell velocity which are responsible for hypoperfusion in the kidney and brain, and that DCLHb improves the perfusion mainly due to an increase in red cell velocity.

Diaspirin crosslinked hemoglobin (DCLHb™), a hemoglobin-based blood substitute, improves regional blood circulation and systemic hemodynamics in normal and hemorrhaged rats. We have conducted extensive studies to determine the pharmacological mechanism responsible for the efficacy of DCLHb in hemorrhaged rats (Chapter 13). Hemorrhage was induced in male urethane anaesthetized rats by bleeding them at a rate of approximately 0.5 to 1 ml/min to a mean arterial pressure of 35-40 mmHg, and maintained at this pressure until a base deficit of less than -12 mmol/l was achieved. Plasma cGMP was found to be 0.10 ± 0.01 pmoles/ml at the baseline and significantly increased following hemorrhage to 0.81 ± 0.17 pmoles/ml at 15 min, 1.43 ± 0.28 pmoles/ml at 30 min and 2.37 ± 0.11 pmoles/ml at 60 min of hemorrhage. Ringer's lactate (4 ml/kg, i.v.) administered as a vehicle produced only a 25%

decrease in plasma cGMP concentration 30 min after its administration to hemorrhaged rats and they survived for <60 min. DCLHb (400 mg/kg, i.v.) on the other hand, produced a significant (-60%) decrease in the plasma concentration of cGMP in hemorrhaged rats. Resuscitation with DCLHb (100 mg/kg, i.v. in a 10% w/v solution) significantly improved oxygen consumption and base deficit at 30 min following resuscitation. These rats survived for >200 min. Resuscitation with NO synthase (NOS) inhibitor, L-NAME (2 mg/kg, i.v. or 10 mg/kg, i.v.), did not produce any improvement in oxygen consumption or base deficit and these rats survived for <90 min. Pretreatment with L-NAME (2 mg/kg, i.v.) significantly attenuated the DCLHb-induced improvement in survival time (<150 min), base deficit, and regional blood flow. Since, DCLHb decreases plasma cGMP levels in hemorrhaged rats, and it is not as effective in rats pretreated with NOS inhibitor, these data suggest that removal by DCLHb of NO formed following hemorrhage contributes toward its efficacy. However, NOS inhibition alone is not effective in the resuscitation of hemorrhaged rats, suggesting the involvement of mediators other than NO. It was found that DCLHb increases plasma endothelin (ET) levels and FR139317 (4 mg/kg, i.v.), an ET_A receptor antagonist, significantly attenuated the DCLHb-induced improvement in time of survival, base deficit, systemic hemodynamics and regional blood circulation in hemorrhaged rats. DCLHb-induced increases in ET-1 appear to be contributing toward its efficacy in hemorrhaged rats. It is concluded that following hemorrhage, an increase in NO and ET-1 takes place. The removal of NO by DCLHb contributes in decreasing the toxicity due to NO, while an increase in ET-1 following hemorrhage appears to be a compensatory response which is augmented by DCLHb.

14.2 Implications for future research

Hemoglobin based blood substitutes will provide for the first time oxygen carrying capabilites in the management of critical care patients. The word hemoglobin based blood substitutes is a misnomer and we think the correct word should be hemoglobin based therapeutic agent. The reason for this is that following identification of pharmacological properties of DCLHb the use of this agent is not simply to substitute blood for oxygen carrying capabilites but to develop other innovative applications for this purified and modifed hemoglobin solution. A variety of potential applications of DCLHb are currently under investigation. Some of the potential indications are (1) restoration of oxygen delivery, even prior to transport to the hospitals, in trauma resuscitation to prevent or reversal shock and subsequent end organ failure, (2) reduction in the degree of ischemia following stroke or myocardial infarction, (3) improvement of tissue perfusion in septic shock, cardiogenic shock, post-surgical shock, (4) improving the oxygenation of tumors so that they are more susceptible to radiation and chemotherapy, (5) reduction of red cell sickling and improve blood rheology during crisis in

sickle cell anemia, (6) perfusion of oxygen hemoglobin solutions into the occluded coronary artery through balloon angioplasty catheters, (7) hemodilution in patients undergoing elective surgery, and (8) extracorporeal oxygenation like cardiopulmonary bypass and organ perfusion.

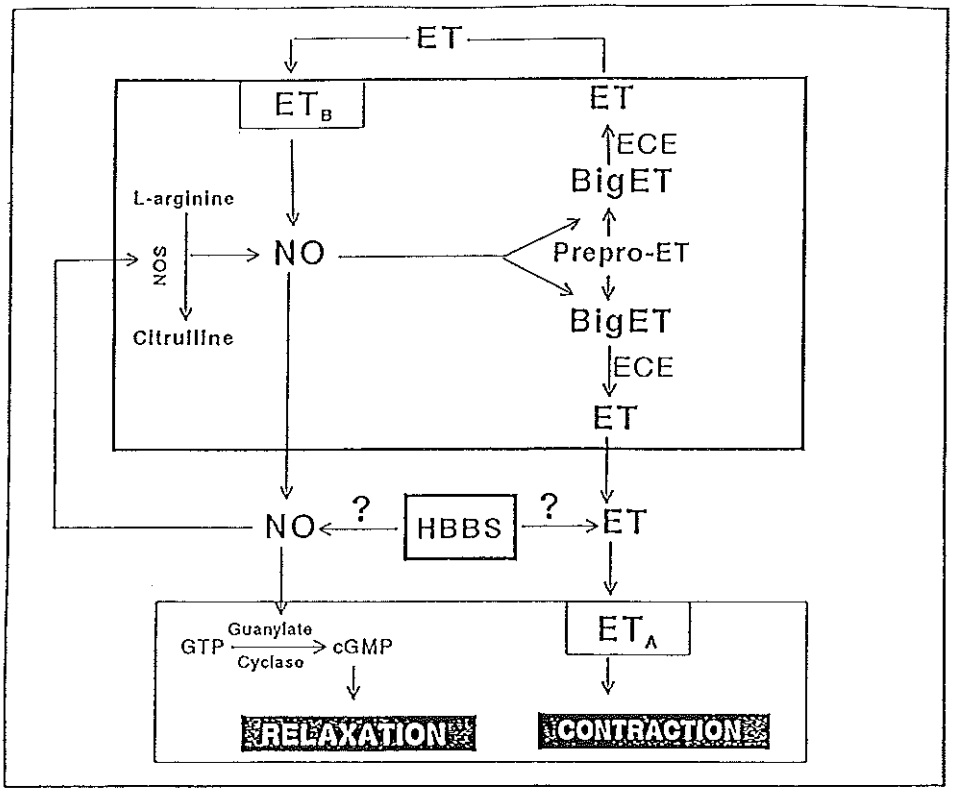

Fig. 2 Shows the role of endothelin (ET) and nitric oxide (NO) in maintaining the homeostatic mechanisms in the wall of blood vessels. ET produces vasoconstriction by inducing smooth muscle contraction due to its action on ET_A type of receptors located on the smooth muscle cells. This is counterbalanced by the action of NO on guanylate cyclase and increasing the production of cGMP, which produces relaxation of the smooth muscles. The production of NO is also regulated by ET acting on ET_B type of receptors located on the luminal side of endothelial cells. It is our hypothesis that both ET and NO contribute to the pathophysiology of hemorrhagic shock. Following severe hemorrhage loss of vascular tone occurs due to an increase in NO and cGMP levels and body tries to compensate this by increasing circulating ET levels. Therefore, if low doses of ET agonists are administered along with NO radical scavengers or NO inhibitors an effective low volume resuscitation can be achieved.

14.3 Impact of haemoglobin therapeutic agent on critical care medicine

Several types of hemoglobin-based blood substitutes have been developed and are in different phases of clinical trials. Studies have demonstrated the efficacy of hemoglobin-based blood substitutes in resuscitation following hemorrhage in animals. An ultrapurified, polymerized, bovine hemoglobin solution has been shown to improve the cerebral blood flow when administered to hemorrhaged rats. Stroma free polymerized bovine hemoglobin has been found to be effective in restoring the systemic hemodynamic parameters in hemorrhaged dogs. Another study using polymerized ultrapurified bovine hemoglobin has shown that it is effective in restoring the cardiovascular parameters following ovarian hemorrhage in a miniature horse. A liposome encapsulated hemoglobin preparation consisting of lyophilized powder for use as "instant blood" has also been described. The use of hemoglobin-based therapetuic agents will introduce a new approach in critical care medicine of not only improving perfusion but delivering oxygen to tissues.

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Summary

Viral contaminations, recurring shortages, cross-matching, typing, limited shelf life and problems in storage are some of the good reasons for developing new oxygen carriers. The hemoglobin tetramer has the property of carrying, delivering and releasing oxygen in a cooperative manner, and has the ability to achieve high saturation at ambient oxygen pressures. Although the attempts to develop an oxygen-carrying alternative to red blood cells have spanned the last 100 years, however, it has proven difficult to develop a clinically useful hemoglobin-based oxygen carrier. The major problems have been that hemoglobin outside the red blood cell has an increased affinity to oxygen and dissociates into dimers and monomers. Attempts have made to overcome these limitations by using hemoglobin molecule either as stroma-free hemoglobin, modified stroma-free hemoglobin or liposome-encapsulated hemoglobin. Diaspirin cross-linked hemoglobin (DCLHb) is a modified hemoglobin solution derived from human erythrocytes. DCLHb was one of the first blood substitute put to test in human clinical trials in Europe and United States. Three Phase II studies with DCLHb were successfully completed in the United States, Belgium and the United Kingdom in 1995, and now a pivotal efficacy trial is underway in Europe and the FDA is reviewing the protocol of a planned Phase III trial in United States.

DCLHb presently is in advanced stages of clinical trial, it is therefore important to know its mechanism of action. The purpose of the present study was to determine the cardiovascular actions of DCLHb in normal and hemorrhaged situations and to investigate the pharmacological mechanism of action of DCLHb.

The present study describes for the first the mechanism of action of DCLHb in normal and hemorrhages rats. It was found that purification and chemical modification of hemoglobin alters its cardiovascular actions. The cardiovascular actions of DCLHb were found to be attenuated by α-adrenergic receptor antagonists, prazosin and yohimbine. Although adrenergic mechanisms are involved in the cardiovascular actions of hemoglobin solutions, but the effect of DCLHb on regional blood circulation was found to be extremely different from that produced by equipressor doses of norepinephrine. The results of this thesis clearly indicate that DCLHb is not a simple oxygen carrying solution but has significant pharmacological properties.

It was found that DCLHb in a dose dependent manner produced significant improvement in survival time, oxygen consumption, systemic hemodynamics, and regional blood circulation of hemorrhaged rats. Using laser Doppler flowmetry, it was found that following hemorrhage it is the decrease in concentration of moving red blood cells (CMBC) and red cell velocity which are responsible for hypoperfusion in the tissues, and that DCLHb improves the perfusion mainly due to an increase in red cell velocity. The efficacy of DCLHb in hemorrhaged situation has been

attributed to its ability to transport oxygen, however, the present study demonstrates that the ability of DCLHb to produce significant alterations in the cardiovascular system also contributes toward its efficacy. The role of NO and ET in hemorrhagic shock has been elucidated as a result of the present studies.

Plasma cGMP was found to be significantly increased following hemorrhage. Ringer's lactate administered as a vehicle produced only a 25% decrease in plasma cGMP concentration after its administration to hemorrhaged rats and they survived for <60 min. DCLHb on the other hand, produced a significant decrease in the plasma concentration of cGMP in hemorrhaged rats and these rats survived for >200 min. Resuscitation with NO synthase (NOS) inhibitor, L-NAME, did not produce any improvement in oxygen consumption or base deficit and these rats survived for <90 min. Pretreatment with L-NAME significantly attenuated the DCLHb-induced improvement in survival time (<150 min), base deficit, and regional blood flow. Since, DCLHb decreases plasma cGMP levels in hemorrhaged rats, and it is not as effective in rats pretreated with NOS inhibitor, these data suggest that removal by DCLHb of NO formed following hemorrhage contributes toward its efficacy. However, NOS inhibition alone is not effective in the resuscitation of hemorrhaged rats, suggesting the involvement of mediators other than NO. It was found that DCLHb increases plasma ET levels and FR139317, an ET_A receptor antagonist, significantly attenuated the DCLHb-induced improvement in time of survival, base deficit, systemic hemodynamics and regional blood circulation in hemorrhaged rats. DCLHb-induced increases in ET-1 appear to be contributing toward its efficacy in hemorrhaged rats. It is concluded that following hemorrhage, an increase in NO and ET-1 takes place. The removal of NO by DCLHb contributes in decreasing the toxicity due to NO, while an increase in ET-1 following hemorrhage appears to be a compensatory response which is augmented by DCLHb.

The present study demonstrates for the first time that in hemorrhagic shock, ET release contributes toward recovery from shock. ET like compounds could be used clinically in the management of hemorrhagic and other types of shock.

Samenvatting

Virale contaminatie, veel voorkomende tekorten, kruistypering, beperkte houdbaarheid en problemen met de opslag van het bloed zijn enkele van de goede redenen om nieuwe zuurstofdragende media te ontwikkelen. Het hemoglobine tetrameer heeft eigenschappen om zuurstof te binden, af te leveren en los te laten op een coöperatieve manier, en kan een hoge saturatiegraad bereiken bij omgelegen zuurstofdruk. Ondanks dat er al honderd jaar pogingen worden ondernomen om een zuurstofdragend alternatief te vinden voor erythrocyten, is het moeilijk gebleken een klinisch bruikbare zuurstofdrager, die op hemoglobine gebaseerd is, te ontwikkelen. De grootste problemen ontstonden doordat hemoglobine buiten de erythrocyt een verhoogde affiniteit voor zuurstof vertoont en snel dissocieert in mono- en dimeren. Gepoogd is deze beperkingen te omzeilen door stroma-vrij hemoglobine, gemodificeerd stroma-vrij hemoglobine of hemoglobine ingekapseld in liposomen te gebruiken. Diaspirin cross-linked hemoglobin (DCLHb) is een gemodificeerde hemoglobine-oplossing verkregen uit menselijke erythrocyten. DCLHb is als één van de eerste bloedvervangende middelen getest in patiënt-gebonden klinische trials in Europa en in de V.S. Drie fase II studies in de V.S., België en het Verenigd Koninkrijk zijn succesvol afgesloten in 1995 en voorbereidingen voor een 'pivotal efficacy' trial in Europa worden nu getroffen en het protocol van een in de V.S. geplande fase III studie wordt door de FDA beoordeeld.

Omdat DCLHb in een vergevorderd stadium van klinische trials is, is het van belang het werkingsmechanisme te leren kennen. Het doel van de huidige studie was de cardiovasculaire effecten van DCLHb in normale en adergelaten toestand vast te stellen en het farmacologische mechanisme te onderzoeken.

De huidige studie beschrijft voor het eerst het werkingsmechanisme van DCLHb in normale en adergelaten ratten. Gevonden werd dat purificatie en chemische modificatie van hemoglobine de cardiovasculaire effecten verandert. De cardiovasculaire effecten van DCLHb werden verminderd door de α-adrenoceptor antagonisten prazosine en yohimbine. Hoewel adrenerge mechanismen betrokken zijn bij het cardiovasculaire effect van hemoglobine-oplossingen, bleek het effect van DCLHb op de regionale bloedcirculatie zeer verschillend te zijn van dat geproduceerd door equipressor doseringen van noradrenaline. De resultaten van dit proefschrift wijzen er duidelijk op dat DCLHb niet een eenvoudige zuurstofdragende oplossing is, maar over belangrijke farmacologische eigenschappen beschikt.

DCLHb verbeterde dosis-afhankelijk en significant de overlevingstijd, zuurstof consumptie, systemische hemodynamiek en regionale bloedcirculatie van adergelaten ratten. Met laser Doppler flowmetrie werd gevonden dat na aderlating de afgenomen concentratie van bewegende erythrocyten en afgenomen snelheid van de erythrocyten verantwoordelijk zijn voor hypoperfusie in de weefsels, en dat DCLHb de perfusie met name verbetert door een toename van de erythrocyten snelheid. De werkzaamheid van DCLHb bij aderlating is voorheen toegeschreven aan het zuurstoftransporterend vermogen, maar de huidige studie laat zien dat het vermogen van DCLHb om significante veranderingen tot stand te brengen in het cardiovasculaire systeem ook een bijdrage levert. De rol van nitriek oxyde (NO) en endothelinen (ET) in hemorrhagische shock is verhelderd door deze studies.

Plasma cGMP was significant verhoogd na aderlating. Toediening van Ringer's lactaat als een vehikel aan adergelaten ratten resulteerde in een afname van 25% van cGMP en de ratten overleefden minder dan 60 minuten. DCLHb, anderzijds, produceerde een significante afname van cGMP en de ratten overleefden langer dan 200 minuten. Reanimatie met de NO synthase (NOS) remmer, L-NAME, had geen verbetering van de zuurstofconsumptie of base deficiet tot gevolg en deze ratten overleefden korter dan 90 minuten. Voorbehandeling met L-NAME deed de door DCLHb geïnduceerde verbetering in overlevingstijd (<150 min), het base deficiet en regionale bloeddoorstroming significant afnemen. Het feit dat DCLHb de plasma cGMP concentratie vermindert in adergelaten ratten en minder effectief is bij ratten die voorbehandeld zijn met een NOS remmer, doet vermoeden dat wegvangen door DCLHb van het na bloeding gevormde NO bijdraagt aan de effectiviteit. Maar NOS inhibitie alleen is niet effectief bij de reanimatie van adergelaten ratten. Dit doet de betrokkenheid van meer mediatoren dan NO alleen vermoeden. DCLHb deed plasma ET toenemen en FR139317, een ET_A receptor antagonist, deed de door DCLHb bewerkstelligde verbetering in overlevingstijd, base deficiet, systemische hemodynamiek en regionale bloeddoorstroming significant afnemen. De DCLHb geïnduceerde toename van ET-1 lijkt bij te dragen aan de effectiviteit bij adergelaten ratten. Er wordt geconcludeerd dat NO en ET-1 na aderlating toenemen. De verwijdering van NO door DCLHb vermindert de toxiciteit van NO, terwijl de toename van ET-1 na aderlating een compensatoir effect lijkt te zijn, dat wordt versterkt door DCLHb.

Deze studie toont voor de eerste keer aan dat in hemorrhagische shock, vrijmaking van ET bijdraagt aan het herstel. ET-achtige verbindingen zouden in de kliniek gebruikt kunnen worden bij de behandeling van hemorrhagische en andere typen shock.

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