EFFECTS OF MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY ON TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS AND LEUCOCYTE-INDUCED HAEMOLYSIS IN DOGS

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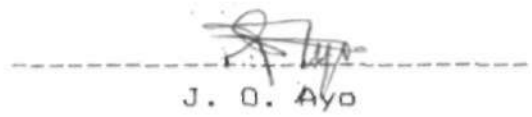
AUGUST, 1989

DECLARATION

I hereby declare the originality of this work carried out by me in the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria under the supervision of Professor Y. O. Aliu, Drs. P.M. Rahamathulla and I. O. Adeoshun.

The work of other investigators referred to is duly acknowledged. No part of this thesis has been previously submitted for a degree or diploma.

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THESIS APPROVAL

This thesis by Dr. Joseph Olusegun AYO meets the regulations governing the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to scientific knowledge and literary presentation.

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ABSTRACT

Eighteen dogs were experimentally induced with acute myocardial ischaemia or reperfusion injury, while seven dogs with sham operation served as controls. Rectal temperature, respiratory and heart rates as well as total and differential leucocyte counts were examined 24 hours before and after surgery. Leucocytes harvested from blood samples obtained from each dog 24 hours pre- and post-surgery were examined for their capacity to induce haemolysis against sensitised caprine erythrocytes in the presence of phorbol myristate acetate (PMA). Statistically significant ($P<0.05$) post-surgical increase in rectal temperature, respiratory rate and heart rate as well as neutrophilia were obtained in the experimental dogs. Sham operation resulted in insignificant increase in rectal temperature, respiratory rate, heart rate and in neutrophilia which might be due to the effect of anaesthesia, surgery and trauma. Highly significant ($P<0.01$) increase in leucocyte-induced haemolysis was obtained in experimental but not in sham-operated dogs, either with or without PMA. Tachycardia, tachypnoea and neutrophilia are compensatory mechanisms aimed at restoring normal cardiovascular activity and, consequently, myocardial oxygen and nutrient supply. Leucocyte-generated oxygen-derived free radicals might be involved in the leucocyte-induced haemolysis. This cytotoxic potential of leucocytes may be an additional factor contributing to myocardial ischaemia-reperfusion injury.

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CHAPTER 1

INTRODUCTION

Coronary artery disease (CAD) manifested by myocardial ischaemia-reperfusion injury and its myriad manifestations is acquiring increasing significance in Nigeria, especially among the urban dwellers (Carlisle and Wilhelmson, 1972; Bwala, 1989).

It is the largest single cause of morbidity and mortality of the western world. For example, death rate from ischaemic heart disease in the United States is approximately 35 percent of all human deaths, a three-fold increase over death rates attributable to all forms of cancer and about 33 percent of all deaths. Of the survivors, 50 percent will develop significant angina pectoris, face a ten-fold increase in risks of left ventricular failure, four-fold increase in risk of sudden death, six percent annual recurrence rate and four percent annual mortality (Kannel, 1976; Borrow et al., 1978). Economic losses incurred due to CAD in U.S.A. alone runs into billions of dollars a year (Epstein, 1969).

As early as 1698 before myocardial infarction in man was recognised at autopsy by Weigert (1880), experimental coronary artery occlusion was attempted in dogs by Thirac. The latter recorded the first pathologic description of myocardial injury; it was noted that the heart stopped beating immediately. Today, the increasing accuracy of the diagnosis of acute myocardial injury by invasive and non-

invasive techniques, estimation of myocardial injury size in experimental animals and man are acquiring increasing accuracy. Diagnosis of myocardial injury and modification of its size by a variety of therapeutic measures have become major research efforts in cardiology throughout the world.

In domestic animals, myocardial injury is often associated with diseases of various aetiologic factors, including:

1. bacterial infections such as colibacillosis, salmonellosis, pasteurellosis, leptospirosis and swine erysipelas.
2. viral diseases such as equine infectious anaemia and foot-and-mouth disease.
3. parasitic diseases such as sarcocystosis.
4. canine leukaemia and
5. neurosa (Sudakov, 1985).

Inflammation is a feature in these diseases, associated with increase in leucocytic accumulation. Leucocytic infiltration in a number of cardiomyopathies is also associated with myocardial injury such as round cell disease of turkeys (Staley et al., 1981), inflammatory myocarditis (Hastillo et al., 1982) and Chagas's disease (Sadigursky et al., 1982). However, the mechanisms linking leucocyte accumulation in blood and infiltration into interstitial tissues to myocardial injury remain to be fully elucidated.

One of the mechanisms proposed is that lysosomal enzymes in the granules of these leucocytes reduce molecular

oxygen with release of oxygen-derived free radicals and metabolites such as hydroxyl radical (OH•), superoxide radical (O_2^-), hydroxyl ion (OH^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) into the extracellular spaces. These oxygen-derived free radicals and metabolites are involved in cell and tissue injury (Babior et al., 1973; Fong et al:, 1973; Root and Metcalf, 1977; Fantone and Ward, 1982; Freeman and Crapo, 1982) associated with a variety of disease states, including ischaemia-reperfusion injury, emphysema, adult respiratory distress syndrome, immune complex-mediated vasculitis, and rheumatoid arthritis (Fantone and Ward, 1985).

Diagnostic tests have been developed based on free radical approach to the pathogenesis of some diseases of various aetiologic factors such as diabetes mellitus (Kulikova et al., 1986), pasteurellosis (Kadoshnikov et al., 1986), viral hepatitis (Mironov et al., 1986), tuberculosis (Shutskaya et al, 1986), echinococcosis (Asanov et al., 1986 and cancer (Pavlyuk, 1986) as well as avitaminosis E (Smolinskaya et al., 1986).

Also, oxidation is a major contributor to the degenerative processes that lead to ageing and cellular breakdown (Dormandy, 1969). This is particularly true of the erythrocyte which is at high risk due to exposure to high oxygen concentrations and its inability to replace damaged components by re-synthesis. Consequently, the erythrocyte devotes much of its metabolic activity to reductive processes that combat oxidation threat. When

these reductive processes are deficient or overwhelmed, oxidation of erythrocyte constituents will occur with consequent haemolysis. This is the basis of a characteristic group of diseases, the oxidative haemolytic anaemias, induced by oxygen-derived free radicals (Gordon-Smith and White, 1974; Carrel et al., 1975).

In view of the above, it is logical to investigate the effect of leucocyte-generated, oxygen-derived free radicals in vitro on a simple cell type like the erythrocyte. The degree of haemolysis induced will serve as an indicator of free radicals generated by the stimulated leucocytes, thus mimicking the mechanisms underlying the destruction of cardiac myocytes in vivo by oxygen-derived free radicals. It is conceivable that the evidence for central role of oxygen-derived free radicals may justify the following objectives of the research to:

1. investigate possible changes in some body physiologic parameters in acute myocardial ischaemia-reperfusion injury,
2. investigate mechanisms involved in leucocyte - induced haemolysis using erythrocytes (RBCs) as target cells and phorbol myristate acetate (PMA) as a leucocyte stimulator.
3. elucidate the mechanism of myocardial damage by leucocytes in acute myocardial ischaemia-reperfusion injury.

4. investigate the role of leucocyte in oxidative haemolytic anaemias.
5. develop a rapid, relatively simple and sensitive diagnostic test for ischaemia-reperfusion injury.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Cardiac Circulation

2.1.1 Normal Coronary Blood Flow and its Variations

The main coronary arteries lie on the surface of the heart, and small arteries penetrate into the cardiac muscle mass. Apart from 0.5mm, or less of the inner surface of the mass that can obtain nutrition directly from blood in cardiac chambers, the main coronary arteries are entirely responsible for supply of nutritive blood to the myocardium (Gregg and Fisher, 1963; Guyton, 1981).

In the dog, the right coronary artery supplies mainly the right ventricle, while the left coronary artery supplies mainly the left ventricle and a small part of the right ventricle. Eighty five percent of myocardium receives blood from the left coronary artery, thus the dog has a striking preponderance of left coronary arterial supply (Gregg and Fisher, 1963; Keele et al., 1982).

Most of the venous blood flow which constitutes about 80 percent of total coronary blood flow from the left ventricle leaves by way of coronary sinus. Venous blood from the right ventricle flows mainly through the small anterior cardiac veins, which empty directly into the right atrium and are not connected with the coronary sinus. A small amount of coronary blood flows back into the heart through the thebesian veins, which empty directly into all the heart chambers (Berne, 1964; Katz, 1977).

Coronary blood flow varies from 0.4 to 1.6ml per g. per min., with an average of 1.0ml per g. per min. for the dog (Nolting et al., 1958). In strenuous exercise, the heart increases its cardiac output as much as four- to six-fold, and it pumps this blood against a higher than normal arterial pressure. Thus, the cardiac work output under severe conditions may increase as much as six- to eight-fold. Coronary blood flow increases four- to five-fold to supply the extra nutrients needed by the heart. This increase is not quite as much as the increase in work load, hence the ratio of coronary blood to energy expenditure by the heart decreases. The "efficiency" of cardiac contraction, however, increases to make up for this relative deficiency of blood supply (Haddy, 1969; Sanders et al., 1979).

Blood flow through the capillaries of the left ventricle falls to a low value during systole, which is opposite to the flow in all other vascular beds of the body. This is due to the strong compression of the cardiac muscle around the intramuscular vessels during systole. During diastole, cardiac muscle relaxes completely and no longer obstructs blood flow through the left ventricular capillaries, so that blood now flows rapidly during all of diastole (Sanders et. al., 1979).

Blood flow through coronary capillaries of right ventricle undergoes phasic changes similar to those in coronary capillaries of the left ventricle during the

cardiac cycle. Due to sharper fall in force of contraction of the right ventricle than that of the left ventricle, these phasic changes are relatively mild compared with those in left ventricle (Guyton, 1981).

Blood flow in the coronaries is regulated almost exactly in proportion to the need of cardiac musculature for oxygen. In the normal resting state, about 65 percent of arterial blood oxygen is removed as blood passes through the heart and, because not much oxygen is left in the blood, little additional oxygen can be removed subsequently from blood unless the flow increases. Oxygen lack is, therefore, followed by coronary vasodilation, thus increasing blood flow to the cardiac musculature in proportion to metabolic need for oxygen by the muscle fibres (Haddy, 1969).

When coronary blood flow fails to increase appropriately, myocardial strength diminishes rapidly and drastically, often causing acute heart failure. This relative myocardial ischaemia can cause severe pain called anginal pain and injury (Favaloro et al., 1980; Reimer et al, 1983).

2.1.2 The Cardiac Collateral Circulation

The extent of cardiac damage induced either by slowly developing constriction of the coronary arteries or by sudden occlusion is determined by the degree of collateral circulation that is already developed or that can develop within a short period of time after the occlusion (Schaper and Wusten, 1979).

Although in a normal heart relatively few communications exist among the larger coronary arteries, there are many anastomoses among the smaller arteries of diameter 20 to 250 microns.

When a sudden occlusion occurs in one of the larger coronary arteries, the sizes of the minute anastomoses increase to their maximum physical diameter within a few seconds but do not enlarge further for the next 22 hours (Schaper and Wüsten, 1979). Blood flow via these minute collaterals is less than one-half that needed to keep alive the cardiac muscle that they supply. However, collateral blood flow begins to increase, doubling in amount in the next one to two days, often reaching normal or almost normal coronary supply within about one month. It is because of these developing collateral channels that a patient may recover from various types of coronary occlusion (Schaper and Wüsten, 1979).

It is when the coronary arteries are slowly occluded over a period of many years, rather then suddenly, that the collateral vessels can develop. But when the occlusion develops beyond the limits of even the collateral blood supply to provide the needed blood flow, the heart can no more pump even the normally required amounts of blood flow. This is the most common cause of cardiac failure (Schaper et al., 1971; Schaper and Pasyk, 1976; Schaper and Wüsten, 1979; Parmley, 1985).

2.1.3: Regulation of Coronary Blood Flow

The ability of the heart to adjust its blood supply to the widely varying oxygen needs without the help of nerves and hormones is called autoregulation. The mechanism of regulation of coronary blood flow is largely unknown. Several of the hypotheses proposed include metabolic autoregulation, absence or influence of the autonomous nervous system on the coronary vascular bed.

Metabolic Autoregulation

The oxygen supply to the heart is usually accurately adjusted to its metabolic needs. Whenever cardiac activity increases or the arterial oxygen content decreases, the coronary vessels dilate to accommodate the increased amount of flow. The close connection between myocardial activity and coronary blood flow suggests a link between myocardial metabolism and coronary artery tone (Schaper, 1979b).

According to Berne (1964), this link is adenosine released from the myocardial cell at a rate dictated by the cellular metabolism. Adenosine is a split product of the high-energy adenine nucleotides formed in increased amounts, when the adenosine triphosphate (ATP) turnover is increased or when adenosine diphosphate (ADP) cannot be converted to ATP because of lack of oxygen. Adenosine is a potent vasodilator and can diffuse across the sarcolemma to reach the arterioles (Schaper, 1979b).

Oxygen as a Regulator of Coronary Blood Flow

Increased oxygen damand and reduced arterial oxygen content result in increased coronary blood flow, suggesting that oxygen plays a role in autoregulation of the heart (Schaper, 1977b.) Vasoconstriction and vasodilation are believed to be reflection of the sum of duty cycles of the pre-capillary sphincters. Coronary system is finely tuned to the oxygen needs of the heart. It is equipped with a feedback mechanism involving pre-capillary sphincters that sense the oxygen need of the tissues and adjust vascular tone accordingly (Schaper, 1979b).

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Regulation by Chemoreceptors

Regulation of coronary blood flow occurs by chemoreceptors stimulated by chemical changes in the blood. Chemoreceptors located in the carotid and aortic bodies continually monitor alteration in partial pressure of oxygen and carbon dioxide, as well as osmolality of the blood. Regulation of coronary blood flow employ either of the following two possible modes of action:

1. Transmitters that are linked to the transduction of chemical energy. Changes in cardiac activity will produce changes in the rate of release of the hypothetical transmitter which may stem from sarcomere sites, mitochondria or the translocation pathway between mitochodria and sarcomeres.
2. Changes in cardiac activity that necessitates larger amounts of oxygen to transiently lower the tissue oxygen tension. Arterial hypoxaemia will transiently interfere with energy transduction or translocation and increase in cardiac

activity will decrease tissue oxygen tension (Schaper, 1979b).

According to Borgers et al.(1971), an oxygen-sensitive chemoreceptor is present in the heart of man, dog and rat. This chemoreceptor is a cell occupying a pericyte-like location close to the pre-capillary arterioles and capillaries with long pseudopod or dendrite-like extensions. These extensions penetrate deep into the extracellular space in between myocardial cells. The chemoreceptor is equipped with a very active 5-nucleotidase: that is, the enzyme that converts adenosine monophosphate (AMP) into adenosine, a potent vasodilator. The long extensions of the cell membrane sense the oxygen tension along the myocardial cells. A drop in oxygen tension may limit conversion of ADP to ATP, transphosphorylation and 5-nucleotidase will produce adenosine which is released directly at the vascular wall. Since these pericytes are often in direct contact with capillaries, which are often equipped with one layer of smooth muscle, a combination of the adenosine hypothesis with the sphincter hypothesis is possible (Schaper, 1979b).

Role of pH, Carbon Dioxide and Osmolarity in Regulation of Coronary Blood Flow.

Ischaemia leads to both tissue acidosis and vasodilation. Acidosis is caused by the end-product of glycoysis: that is, lactic acid. At pH values below 7.4 vasodilation occurs. While vasoconstriction occurs at values above 7,4. Metabolic acidosis produced by physical exercise would double coronary blood and oxygen through increased cardiac output. At pH 7.8, all known

vasodilators including adenosine are completely inactive. At pH values below 7.4, they are maximally active (Kraupp et al., 1967; Schaper, 1979b.).

Increases in carbon dioxide tension of the blood produce marked coronary vasodilation. The rather powerful vasodilator property of carbon dioxide and its moderator influence on pharmacologic agents have prompted Kraupp et al. (1967) to base regulation of coronary blood flow on tissue carbon dioxide. Carbon dioxide produced by cardiac metabolism may be a candidate for metabolic autoregulation (Schaper, 1979b).

Infusion of potassium ions at low concentrations into the coronary system causes only small increments in coronary blood flow, whereas higher concentrations cause vasoconstriction (Berne, 1964). There is no clear-cut dose-response relationship and correlation between either work hyperaemia or hypoxic hyperaemia and potassium release. This indicates that potassium is not a likely candidate for the coupling between oxygen demand and supply. Inorganic phosphate is also a regulator of coronary blood flow. Phosphate may stem from the breakdown of creatine phosphate which vary with cardiac work load (Honigs, 1968; Schaper, 1979b).

Neural Regulation of Coronary Blood Flow

Coronary blood flow depends on the tone of coronary vessels regulated by the autonomic nervous system. Stimulation of the vagus nerve induces a decrease in coronary blood flow, due to bradycardia, and decrease in blood pressure in the aorta. These

decrease the oxygen consumption of the heart. Stimulation of the sympathetic nerve increases coronary blood flow induced by increase in arterial blood pressure and oxygen utilisation produced by adrenaline and noradrenaline. Although these catecholamines considerably increase myocardial oxygen demand, the increase in coronary blood that ensues may not correspondingly be sufficient to meet the oxygen requirements of the heart (Ishimova, 1980). This may result in myocardial ischaemia.

Prostaglandins

Prostaglandins are naturally occurring polyunsaturated fatty acids of ubiquitous distribution. They exist naturally in E, F, A and B series. E and A type prostaglandins are powerful vasodilators in many vascular beds including the coronary arteries (Higgins et al., 1971). According to Alexander et al. (1975), the myocardium transforms arachidonic acid, a precursor of prostaglandins into prostaglandin A under normoxaemic and anoxaemic conditions. These potent dilatators of coronary arteries have, therefore, been suggested as candidates for mediators of coronary blood flow regulation (Schaper, 1979b).

2.2 Pathophysiology of Myocardial Ischaemia-Reperfusion Injury

Immediately after an acute coronary occlusion, blood flow ceases in coronary vessels beyond the occlusion except for small amounts of collateral flow from surrounding vessels. The muscle area that has either zero flow or so little flow that it cannot sustain cardiac muscle function is said to be infarcted and the overall process is called myocardial infarction (Schaper, 1979a).

Severe ischaemia is a reduction in coronary arterial blood flow to less than 15 percent of non-ischaemic flow (Jennings et al., 1975). In canine heart, this develops in subendocardial region of the myocardium when the circumflex artery supplying this region is occluded near its origin. Myocytes will tolerate 15 minutes of ischaemia of this severity; that is, they survive if flow is restored immediately after this interval. The injury induced by up to 15 minutes of ischaemia is, therefore, reversible (Jennings et al., 1960). Conversely, if ischaemia is allowed to persist for 40 minutes before arterial flow is restored, many of the cells in subendocardial region of severe ischaemia die. Cells that do not survive when ischaemia is eliminated undergo phagocytosis and are replaced by scar tissue; thus, the injury is irreversible (Reimer et al., 1983).

By 40 minutes of ischaemia, cell death in subendocardial region extends to within one to two millimetres of the lateral edge of the ischaemic vascular

bed; thus, in this region there is no lateral border zone of myocardium salvageable by reperfusion (Reimer and Jennings, 1979).

In contrast, myocytes in subepicardial region, where collateral blood flow is greater, can be salvaged by reperfusion at 40 minutes, but will undergo infarction if the ischaemia is allowed to persist. Thus, the onset of irreversible ischaemic cell injury does not occur simultaneously in all regions but develops as an expanding transmural wave front of cell death (Reimer et al., 1977; Reimer and Jennings, 1979).

2.2.1 Events During the Reversible Phase of Ischaemic Injury

The onset of ischaemia is accompanied by rapid changes in both myocardial metabolism and function (Jennings et al., 1981). Within the first 10 to 15 seconds after occlusion, the tissue partial oxygen pressure decreases, mitochondrial respiration is inhibited, and myocytes begin to use anaerobic glycolysis as the principal source of new high-energy phosphate (HEP). Consequently, cellular glycogen levels decrease and lactate begins to accumulate in the ischaemic tissue (Reimer et al., 1981).

Contractile activity stops within ten to fifteen seconds of the onset of severe ischaemia. Thus, ischaemia induces an almost immediate effect on contractile function at a time when all myocytes still are viable and before much adenosine triphosphate (ATP) depletion has occurred. Loss of myocardial contractile function is, therefore, not

necessarily related to irreversibility of ischaemic injury.

Severe ischaemia is associated with progressive loss of cellular pool of HEPs, even though utilisation of HEP by contractile activity is reduced or absent, for two reasons. First, anaerobic glycolysis can produce only three ATP molecules per glucosyl unit of glycogen converted to lactate, versus the 38 molecules of ATP produced with complete aerobic metabolism of glucose. Secondly, after a brief burst of lactate production, the rate of anaerobic glycolysis is limited by end-product inhibition at enzymatic steps. In part, the increasing lactate/pyruvate ratio slows the conversion of pyruvate to lactate such that the ratio of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD) increases and glycolysis becomes inhibited at the glyceraldehyde-3-phosphate dehydrogenase level (Neely and Mogan, 1974). In addition, phosphofructokinase is inhibited by acidosis and high lactate concentration (Gelet et al., 1969; Williamson et al., 1976).

The metabolic consequences of severe ischaemia result both from the reduction in substrate and oxygen delivery and from the failure to remove end products of metabolism. In this regard, severe ischaemia differs from hypoxia or mild ischaemia. In the latter two conditions, metabolites are removed from the tissue by continued perfusion. Due to this difference, the rate of anaerobic glycolysis is much faster in hypoxia with continued flow than it is in severe ischaemia (Rovetto et al., 1973; Neely et al., 1975).

2.2.2 Events Associated with the Onset of Irreversible Myocyte Injury

Features of ischaemic myocardium that are not present in reversibly injured myocytes, but which appear at about the time of the onset of irreversible injury are:

1. Mitochondria which are swollen and contain amorphous matrix densities (Jennings et al., 1965).
2. ATP, which is almost completely depleted (Jennings et al., 1978).
3. Sarcolemmal damage: small breaks in the sarcolemma are seen by electron microscopy (Jennings et al., 1978). Membrane defects also can be demonstrated in vitro by incubating slices of injured myocardium in an oxygenated Krebs-Ringer's phosphate medium. In such slices, intracellular components such as creatine are lost and the inulin-diffusion space expands, indicating increased membrane permeability to inulin and, consequently, irreversible state of ischaemic injury (Reimer et al., 1981).

Reperfusion of irreversible injured cells in vivo results in explosive swelling of cardiac myocytes associated with massive influx of calcium ions (Ca^{2+}) (Kloner et al., 1974; Whalen et al., 1974).

If the irreversibly injured myocardium is reperfused in vivo or re-oxygenated in vitro, rapid changes occur which

are termed explosive cell swelling (Kloner et al., 1974) or reperfusion injury (Rahamathulla and Ashraf, 1984).

The potential relationships between ATP depletion, Ca^{2+} influx, and sarcolemmal damage form the basis of cellular damage and death. The ATP of ischaemic myocytes decreases because ATP utilisation exceeds the capacity of anaerobic glycolysis to produce HEP. Intracellular Ca^{2+} (Ca_i^{2+}) may increase either because of increased membrane permeability to Ca^{2+} or because of inadequate ATP to support the mechanism responsible for Ca^{2+} extrusion from the cell or sequestration of Ca^{2+} by the sarcoplasmic reticulum.

Increased Ca_i^{2+} also may accelerate ATP depletion through Ca^{2+} activated ATPases. Sarcolemmal damage with large subsarcolemmal blebs occurs at ATP levels of three to eight percent of control. The sarcolemma is severely damaged, often with areas in which scattered vesicles are the only sarcolemmal remnants still attached to the glycocalyx. Thus, severe membrane damage has been shown to form the basis of events leading to sarcolemmal damage (Jennings and Reimer, 1981; Reimer et al., 1983).

Numerous hypotheses, which often are interrelated, have been proposed to explain the transition to irreversible myocardial injury (Jennings, 1981). The most popular hypothesis focuses on membrane damage. The lethal event is considered to be disruption of the sarcolemma and perhaps other membrane systems of the cell; accordingly, the proximate cause of irreversibility could be the sequence of

events that lead to functional and structural disruption of the membranes.

The two major consequences of sarcolemmal damage are the loss of critical intracellular components such as enzymes and co-factors through a leaking membrane to the extracellular space (Kaitenbach and Jennings, 1960), and entry of excess Ca^{2+} into the myocyte from the extracellular space, with resultant disruption of much of the internal metabolic machinery of the cell (Jennings et al., 1975; Nayler, 1981).

The causes of membrane disruption have been proposed to include activation of endogenous phospholipases of the sarcolemma as a consequence either of increased intracellular Ca^{2+} or of depressed phosphorylation of membrane proteins by the reduced sarcoplasmic ATP of the ischaemic cell (Jennings et al., 1978; Rahamathulla et al., 1983; Ashraf and Rahamathulla 1984; Rahamathulla and Ashraf, 1984).

Alternatively, membranes could be damaged because of free radical effects such as liperoxidation (Recknagel, 1967), the action of phospholipases released from lysosomes, the detergent action of acyl-carnitine and acyl-coenzyme A, which accumulate in ischaemia (Neely and Feuvray, 1981), or failure to resynthesise membrane lost through pinocytosis or other processes (Reimer et al., 1983).

Early therapeutic intervention with beta-adrenergic blockers, such as propranolol (Rahamathulla et al., 1983; Ashraf and Rahamathulla, 1984), and timolol (Anonymous,

1984a), as well as calcium channel blockers such as diltiazem (Antman et al., 1980; Rahamathulla et al., 1983; Ashraf and Rahamathulla, 1984), verapamil (Antman et al., 1980; Anonymous, 1984b), and nifedipine (Pearle, 1984) yielded little success.

In fact, in one study conducted in baboons subjected to two hours of left anterior descending coronary artery occlusion with and without nifedipine administration, no reduction in infarct size was observed (Geary et al., 1982).

Intracoronary infusion of streptokinase as thrombolytic therapy in acute myocardial infarction has been observed to be of little value. Streptokinase infusion, apart from being of little therapeutic effect, is associated with the risk of angioraphy in the setting of acute myocardial infarction, reocclusion and bleeding; and such secondary interventions as angioplasty or bypass surgery must be considered (Rentrop et al., 1984).

2.3 Leucocytes and Their Function

Leucocytes or the white blood cells are the mobile units of the body's protective system. They, unlike the erythrocytes, are complete (nucleated) cells of the blood with an average life span of about 14 days. There are two types of leucocytes in the body: granulocytes and agranulocytes. The granulocytes or the polymorphonuclear leucocytes have granular appearance and they are made up of neutrophils, eosinophils and basophils. The agranulocytes comprise monocytes, lymphocytes and plasma cells. The

granulocytes, monocytes and a few lymphocytes are formed in the bone marrow, while the lymphocytes and plasma cells are formed in the lymphogenous organs such as the lymph glands, spleen, thymus and tonsils (Smirnov et al., 1981).

After formation, leucocytes are specifically transported to areas of serious inflammation, thereby providing rapid and potent body defence mechanism against invading organisms by either ingesting them — that is, by the process of phagocytosis, or attaching to specific invading organisms and destroying them (Rossi et al., 1985).

The concentration of the different types of leucocytes in the blood varies from one species of animals to the other. The adult dog, for example, has approximately 8-18 x 10^3 leucocytes per cubic millimetre of blood. The normal range, in percentages, of different types of leucocytes are as follows: neutrophils, 43-71; basophils, 0-1; monocytes, 1-5; lymphocytes, 21-40 and eosinophils, 3-9 (Smirnov et al., 1981).

The life span of the granulocytes in the blood is about twelve hours, while that of the monocytes is difficult to assess since they wander back and forth between the tissues and blood. Monocytes, however, may live for weeks or even months, especially in tissues unless they are destroyed while combating infections or inflammatory processes. The average life span of lymphocytes is 100 to 300 days, or in some instances perhaps even years depending on tissue's need for them (Brecher et al., 1962; Smirnov et al., 1981).

2.3.1. Properties of Leucocytes:

2.3.1.1 Diapedesis

This is the process whereby leucocytes squeeze through the pores and even endothelial cells of the blood vessels.

2.3.1.2 Amoeboid Motion

Once leucocytes have entered tissue spaces, they move through the tissues by amoeboid motion, moving at least three times their own length each minute.

2.3.1.3. Chemotaxis

This is the property whereby leucocytes move either towards or away from a chemical (Guyton, 1981). Chemotactic substances are divided into two groups: Cytotaxins and cytotaxigens. Cytotaxins are substances capable of attracting leucocytes directly into the site of injury. Cytotaxigens are on their own not chemotactic but they enhance the conversion of non-chemotactic agents to cytotaxins. For example, staphylococcal protein A has a strong chemotactic attraction in vivo to rat leucocytes (Onyia, 1988). Staphylococcal protein A reacts with the Fc portion of immunoglobin molecule G (IgG) to produce the split product C5a (Kronvall and Gewruz, 1979).

Cytotaxins for leucocytes include C3a and C5a components of the complement, kallikrein, denatured protein and bacteria. Neutrophil cytotaxigens include trypsin, plasmin, collagenase, antigen-antibody complexes and bacteria toxins. The mechanism of leucotaxis involves the activation of

physical and chemical processes which decrease the surface tension of leucocytes. This leads to the formation of pseudopodia directed towards the stimulus and contraction of actomyosin filaments of the pseudopodium. Leucotaxis is associated with increase in oxygen utilisation by the leucocytes. Neutrophils migrate to the site of inflammation through the tissue between the endothelial cells (Ado, 1980; Savoysky, 1985). Granulocytes take from two to 12 minutes to penetrate the vascular wall and thereafter they may move through a clear space in the tissue at about 20 microns in one minute (Florey, 1970). Mononuclear leucocytes migrate by penetrating through the endothelial cells into the basal membrane from where they move out into the inflamed tissue. This emigration is much slower than that of the neutrophil. By this active migration, leucocytes pass out of the vessels into the surrounding tissue and continue their movements (Florey, 1970; Ado, 1980).

In acute inflammation, granulocytes migrate in far greater numbers than monocytes and lymphocytes (Florey, 1970). With the passage of time, the granulocyte ceases to be the commonest cell in an exudate, and is replaced by the monocyte or macrophage (Florey and Jennings, 1970). Monocytes and lymphocytes migrate to site of inflammation after neutrophils because mononuclear leucocytes are less sensitive to chemotactic agents (Ado, 1980).

2.3.1.4 Phagocytosis

Metchnikoff (1888) demonstrated phagocytosis in worms, arthropods, molluscs, tuna, crustaceans, insects and vertebrates. He considered inflammatory reaction to be a defence mechanism in which phagocytosis of the deleterious agent plays the principal role. Since the initial Metchnikoff's observations in 1887, a large body of information has accumulated defining the roles of phagocytes in acute and chronic inflammatory reactions. When a host tissue is challenged by a pathologic insult of either an immunologic or non-immunologic nature, inflammatory reaction may occur, with subsequent clearance of the pathologic stimulus by the leucocytes. While performing this scavenging role, leucocytes may further damage the surrounding tissue. Tissue injury may, therefore, result from either the direct effects of the pathologic agent or as a consequence of an inflammatory cell influx. Leucocytes play a key role in acute and/or chronic inflammatory responses (Fantone and Ward, 1982; Marklund. 1988).

When pathogenic agents are formed or deposited in host tissues, circulating leucocytes may be recruited from the intravascular compartment to the site of insult by local generation of soluble mediators. The process by which cells migrate from the circulation to an inflammatory site involves the mechanism of directed cell migration and chemotaxis (Ward, 1980).

Leucocytes, especially the neutrophils, trigger membrane phospholipids to release arachidonic acid which is

converted by specific lipoxygenases to the potent chemotactic agents hydroxy-eicosatetraenoic acid (HETE) and leukotriene B_4 (LTB_4). The chemo-attractant LTB_4 promotes further recruitment of neutrophils as well as monocytes into the acutely inflamed region at the site of ischaemic tissue injury. Many of the particulate and soluble phlogistic mediators generated at sites of inflammation activate the metabolic processes of phagocytes, a consequence of which is an increase in the capacity of these cells to ingest pathogenic substances. This activation process may be initiated by the binding of mediators to specific receptors on the cell surface or by the process of phagocytosis of particulate material (Nossal, 1987).

When a foreign particle is deposited in host tissue, it may or may not be coated with specific host proteins such as immunoglobulins and/or complement, depending on its organic make-up. These proteins, called opsonins, can enhance recognition of the particle by phagocytes through binding to specific receptors on the phagocyte cell surface such as Fc receptor and $C3_b$ receptor. After recognition these foreign particles are engulfed by the phagocyte and internalised within cytoplasmic vacuoles, to which are fused lysosomal granules, forming phagolysosomes. These fused granules discharge into the phagocytic vacuole potent enzymes capable of degrading a wide variety of biologic substances such as cytomembranes, collagen, elastin and glycosaminoglycans. The process of fusion of lysosomal granule with phagosome is

called degranulation (Fantone and Ward, 1982).

Glucose provides energy for phagocytosis. It could be used through glycolysis and the hexose monophosphate shunt, or the products of glycolysis can be further utilised through the Kreb's cycle. All these biochemical pathways could yield ATP, which could then be a source of energy for phagocytosis. Studies with metabolic inhibitors suggest that glycolysis is essential to phagocytosis. Thus, addition of iodoacetate or sodium fluoride to the medium prevents phagocytosis, whereas non-metabolic inhibitors such as cyanide and dinitrophenol have no such effect. Since glycolysis is essential for phagocytosis, insulin given together with glucose stimulates phagocytosis (Van Lancker, 1976).

Although glycolysis is essential for phagocytosis, when phagocytosis occurs, other pathways are also stimulated. The rate of respiration and oxidation of the carbon-1 of glucose through the hexose monophosphate shunt is also stimulated (Van Lancker, 1976).

In vitro studies have shown that during phagocytosis, neutrophils, monocytes and tissue macrophages release lysosomal enzymes such as lactoferrin, myeloperoxidase, and nicotinamide adenine dinucleotide oxidase and oxygen-derived free radicals into the external tissue environment (Roos, 1980).

It is conceivable that an acute ischaemic insult may trigger an inflammatory response in which the leucocyte release these lysosomal enzymes extracellularly and which

are capable of proteolytic destruction and liquefaction of injured tissue (Fantone and Ward, 1982).

2.4 Leucocyte and Free Radical Production

In response to activation by particulate and/or specific soluble inflammatory mediators, leucocytes, particularly neutrophils and monocytes undergo a respiratory burst. This is associated with two- to twenty-fold increase in oxygen consumption, increased glucose metabolism via the hexose monophosphate shunt (Sbarra and Karnovsky, 1959; DeChatelet, 1975; Drath and Karnovsky, 1975; Karnovsky et al., 1975; Becker et al., 1979; Fantone and Ward, 1985) and generation of reactive oxygen-derived free radicals and their metabolites (Rossi et al., 1985). During this respiratory burst which is due to activation of a membrane bound NADPH oxidase, this enzyme and the lysosomal enymes in leucocytic granules reduce molecular oxygen to superoxide radical (O_2^-), singlet oxygen [O_2 ($^1\Delta g$)], and hydrogen peroxide (H_2O_2) all of which are released into extracellular spaces. Other highly reactive oxygen-derived free radical and metabolite produced by stimulated leucocytes are the hydroxyl radical ($OH^\cdot$), and hypochlorous acid (HClO), respectively. These oxygen-derived free radicals and metabolites produce significant cell and tissue injury by altering cellular proteins, nuleic acids, membrane lipids, cytosolic molecules and extracellular matrix constituents (Halliwel, 1981; Fantome and Ward, 1982; Freeman and Crapo, 1982; McCord, 1985; Rossi et al., 1985).

The first product of the oxidase is the free radical O_2^-, which rapidly dismutates to H_2O_2.

$$2O_2^- + 2H^+ \xrightarrow[\text{dismutase}]{\text{Superoxide}} H_2O_2 + O_2 \qquad (1)$$

The classical Haber-Weiss' reaction, which is the reaction between O_2^- and H_2O_2 generates the strong oxidant hydroxyl radical (OH·):

$$H_2O_2 + O_2^- \longrightarrow OH\cdot + OH^- + O_2 \qquad (2)$$

The rate constant of this reaction is very small and it could not occur at the low concentration of O_2^- and H_2O_2. But since several transition metal ions, especially iron, are present in significant amounts in living systems, the Haber-Weiss reaction is likely to be catalysed by iron and lactoferrin (Badwey and Karnovsky, 1980; Halliwel et al., 1980):

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

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \qquad (4)$$

Other free radicals may be derived from metal catalysed reaction of O_2^- with peroxide or other organic hydroperoxide to form a variety of oxidising radicals such as alkoxy and acyloxy radical in addition to OH·, which form a family of OH^--like oxidising species. Singlet oxygen [O_2 (^{1} g)] is formed by the metal-catalysed Haber-Weiss reaction or as a product of reaction between H_2O_2 and myeloperoxidase.

The myeloperoxidase-hydrogen peroxide-halide system

generates other toxic molecules such as hypohalous acids (HOX) including hypochlorous acid (HOCl), long lived oxidant N-chloramine (RNHCl) and aldehydes (Sbarra et al., 1977; Weiss et al., 1983). Superoxide anion (O_2^-) may act as an oxidant or as a reducent, but the main effect is linked to the strong oxidant OH°, HOCl and RNHCl, and to the action of myeloperoxidase — hydrogen peroxide-halide system. Although the interactions of these oxidants with many molecular targets cause a series of functional and harmful cellular changes as well as extracellular structures, inflammatory cells are equipped with efficient mechanisms for degradation or inactivation of free radicals and other toxic molecules. The mechanisms include scavengers such as sugar, unsaturated fatty acids, sulphur-containing amino acids, glutathione, vitamins E and C, metals, ubiquinone, and enzymes including superoxide dismutase, catalase and peroxidase (Rossi et al., 1985; Ajimolayev, 1986).

2.5 Free-Radicals

2.5.1 General Characteristics

A "free radical" is any atom, group of atoms or molecule in a particular state with one unpaired electron occupying its outer orbital. A "biradical" is a species containing two unpaired electrons in its outer orbital; for example, molecular oxygen (O_2). A "radical ion" is a free radical with a positive charge [for example, protonated amine radical, H_3N^+] or negative charge [for example, superoxide anion radical, O_2^-]. Whereas an ion depends on

the relationship of the number of electrons to the number of protons, a free radical is a species possessing an unpaired electron in its outer orbital (Del Maestro, 1980; McCord, 1985).

The one electron reduction of molecular oxygen (O_2) results in the formation of superoxide anion radical, O_2^-. The two electron reduction product of O_2 in the fully protonated form is hydrogen peroxide, H_2O_2, while the three electron reduction product of O_2 is the hydroxyl radical, $OH^\cdot$. Hydroperoxide is represented as ROOH; $ROO^\cdot$ represents peroxide, and $RO^\cdot$, alkoxy radical (Del Maestro, 1980).

The lone electron present in the outer orbital of a free radical endows it with very unusual chemical reactivity and physical characteristics. This reactivity is accounted for by the strong tendency of the unpaired electron to interact with other electrons to form an electron pair and thus a chemical bond. Radicals, however, vary greatly in reactivity; for example, the $OH^\cdot$ is so energetic that it reacts almost with the first molecule it collides with. Radical reactions may be of three types:

1. Initiation reaction, in which free radicals are formed as a result of symmetric cleavage of two electron chemical bond. For example,

$$R:R \text{ ---------->} R^\cdot + R^\cdot \quad (5)$$

Free radicals may be initiated by radiolysis, photolysis, homolysis and during oxidation— reduction

reactions (Pryor, 1976).

2. Propagation reaction, involving a free radical transfer in which the site of free radical is altered, but a free radical always results.
3. Termination reaction, which occurs in radical system resulting in free radical removal from propagation pool.

This forms the basis for selective scavenging of free radical species. To accomplish selective scavenging, one uses an appropriate reactant, A, which can react with a radical, R', as shown in the reaction below:

$$A + R' \longrightarrow P' \qquad (6)$$

Increase in the concentration of A results in scavenging of R'. Selective scavenging is very useful in the identification and characterisation of free radical interactions (Del Maestro, 1980).

The possession of parallel electron spin arrangement by molecular oxygen prevents the direct addition of a pair of electrons; one parallel and one antiparallel electron spin, necessitating an electron spin inversion must occur before a bond could form. Since spin inversion process is slow compared to the life time of collisional complexes, the result is that O_2 in its ground state is a relatively weak oxidant. Therefore, if energetically possible, the one electron reduction of O_2, or univalent pathway, would predominate over the two electron reduction or divalent

pathway. The complete reduction of O_2 by the univalent pathway results in the formation of superoxide anion radical, O_2^-, hydrogen peroxide, H_2O_2, and hydroxyl radical, $OH^{\cdot}$, as intermediate products as shown in equation 7:

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} OH^{\cdot} \xrightarrow{e^- + H^+} H_2O \qquad (7)$$

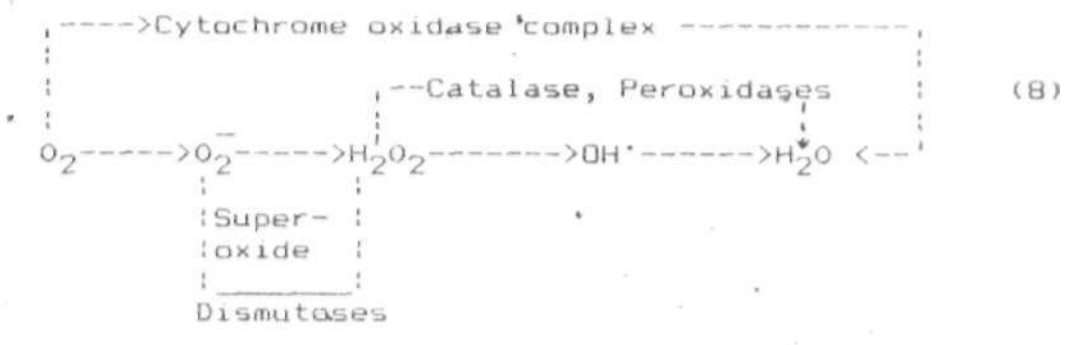
$$H_2O_2 \longrightarrow H_2O$$

These intermediate metabolites are too reactive to be tolerated in living tissues and their removal and control may have been dominant evolutionary pressure with the first appearance of O_2 in the atmosphere. These protective and controlling mechanisms are divided into enzymatic, hydrophobic, hydrophilic and structural groups (Del Maestro, 1980).

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1. Enzymatic Mechanisms:

A variety of enzymatic mechanisms which can bypass the electron spin restriction of oxygen (O_2) to water (H_2O) has evolved. The majority of the O_2 reduced by aerobic cells is carried out tetravalently by cytochrome oxidase (Antonini et al., 1970) which prevents the release of O_2^-, H_2O_2 and $OH^{\cdot}$ into the cellular milieu (Equation 8):



(8)

The O_2^- flux in aerobic cells appears to have necessitated the evolution of a variety of superoxide dismutases whose function is to catalytically scavenge O_2^-. Superoxide dismutase, whose function was discovered by McCord and Fridovich in 1969, catalyses the conversion of O_2^- to H_2O_2 and O_2 (Equation 1).

2. Hydrophobic Mechanism

Hydrophobic regions are those areas within cells in which a set of thermodynamic factors are responsible for the suquestration of hydrophobic or non-polar groups away from H_2O. Lipid membranes with their large content of polyunsaturated fatty acids (Rouser et al., 1968) are the only sustained hydrophobic regions associated with cells.

The lipid peroxidation due to homolytic mechanisms (Pryor, 1976) may occur at low rates in all aerobic cells; but are normally controlled by hydrophobic scavengers such as alpha-tocopherol (vitamin E) which is present in cellular membranes. Vitamin E by providing hydrogen atoms may prevent chain propagating reaction in lipid membranes (Pryor, 1976).

Alpha-tocopherols and beta-carotenes may also quench O_2 ($^1\Delta g$) and thus prevent lipid hydroperoxide formation. Glutathione peroxidase, a selenimum containing enzyme which plays an important role in reducing H_2O_2 to H_2O (Halliwell, 1974, 1977) also reduces lipid hydroperoxides (Del Maestro, 1980).

3. Hydrophilic Mechanism

Hydrophilic protective mechanisms are those chemical substances involved in the ionic or water compartments within the cell. Molecules which may have scavenging potential in ionic environments are compounds such as ascorbic acid, cysteine and reduced glutathione (GSH) (Kosower and Kosower, 1976; Pryor, 1976; Nishikimi and Yagi, 1977). Plasma constituents such as ceruloplasmin and transferrin may also have scavenging capability (Al-Timini and Dormandy, 1977; Gutterbridge 1977; Dormandy, 1978).

4. Structural Mechanisms

The protective mechanisms appear to be intimately associated with the structural integrity of living cells. Disruption of this structural integrity leads very quickly to rancidification — the free radical peroxidation of polyunsaturated lipids (Dormandy, 1978).

Cholesterol, by its structure and size, intercalated into biomembranes may protect fatty acid double bonds from peroxidative injury (Demopoulos et al., 1972). The localisation of certain reactions to peroxisomes and mitochondria and their specialised scavenging mechanisms may also play a role in controlling radical reactions. Low levels of catalase and superoxide dismutase have been found in plasma, cerebrospinal and synovial fluids (McCord, 1974; Salin and McCord, 1977) and by inference in the extracellular space (Del Maestro, 1980).

2.5.2. Leucocyte - Generated Oxygen - Drived Free Radicals and Metabolites, and their Characteristics

2.5.2.1. Superoxide Radical, O_2^-:

A group of cellular enzymes such as xanthine oxidase (Fridovich, 1970) and peroxidases (Halliwell, 1977) are involved in catalysing oxidation reactions which result in the univalent reduction of O_2 to O_2^-. The autoxidation of a large group of compounds including ferredoxin (Misra and Fridovich, 1972) catecholamines (Cohen and Heikkila, 1974) and flavins (Michelson, 1977) also involves O_2^- release. Mitochonria may generate steady fluxes of O_2^- which may also result from the irradiation of living tissues (Petkau et al., 1975; Benon et al., 1977).

The most important reaction that O_2^- may be involved in is its dismutation (Equation 1). In the presence of superoxide dismutase, dismutation may be increased by a factor of 10^9 (Fridovich, 1978a).

An enzyme, NADPH oxidase on the surface of polymorphonuclear leucocytes, macrophages and monocytes reduce O_2 to O_2^-. (Babior et al., 1973), when exposed to appropriate stimuli such as microbes and cell particles using NADPH as an electron donor:

$$2O_2 + NADPH \xrightarrow[\text{oxidase}]{NADPH} 2O_2^- + NADP^+ + H^+ \qquad (9)$$

This is a component of a co-ordinated series of metabolic events called the "respiratory burst" (Babior 1978b).

A significant proportion of the O_2^- generated is released into the extracellular space (Root and Metcalf, 1977), where H_2O_2 formation results from spontaneous dismutation. The interaction of O_2^- with its dismutation product, H_2O_2, resulting in the generation of OH^- is known as the classical Haber-Weiss reaction (Equation 12), while the following equations 10 to 13 are collectively called modified Haber-Weiss reaction or Fenton reaction:

$$Fe^{2+} + H_2O_2 \text{-------} > Fe^{3+} + OH^{\cdot} + OH^- \quad (10)$$

$$OH^{\cdot} + H_2O_2 \text{------} > H_2O + O_2^- + H^+ \quad (11)$$

$$O_2^- + H_2O_2 \text{-------} > O_2 + OH^- + OH^{\cdot} \quad (12)$$

$$Fe^{2+} + OH^{\cdot} \text{----------} > Fe^{3+\cdot} + OH^- \quad (13)$$

Extracellular hydroxyl radical ($OH^{\cdot}$) and possibly singlet oxygen [O_2 ($^1\Delta$ g)] formation may result from an interaction of H_2O_2 and O_2^-. Myeloperoxidase using H_2O_2 and Cl^- as substrates can generate hypochlorite (OCl^-) and subsequently O_2^- ($^1\Delta$ g) (Del Maestro, 1980).

Superoxide (O_2^-) is an oxygen molecule that contains one extra unpaired electron. This extra electron gives O_2^- a net negative charge (12 protons, 13 electrons), while the fact that the extra electron is unpaired characterises the species as a free radical. Superoxide (O_2^-) can be protonated to form the perhydroxyl radical (HO_2) (13 protons, 13 electrons), which is a much stronger oxidant than O_2^-, and may be found in substantial concentrations within the acidic

environment of the phagocytic vacuole.

The ability of O_2^- to reduce or oxidise transition metal ion complexes, to form ligands with metals, to oxidise organic substrates and to be protonated to HO_2 allows this species to play an important role in leucocyte — mediated cytotoxicity (Weiss and LoBuglio, 1982).

2.5.2.2 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide may be formed from two types of reaction:

a) Divalent reduction of O_2 by a group of enzymes such as urate-, D-amino acid-, and glycolate-oxidase. The divalent oxidising enzymes are usually contained within specialised organelles called peroxisomes (de Duve, 1978).

b) Univalent reduction of O_2^- to O_2 (Fridovich, 1970) or autoxidation of various compounds with subsequent dismutation of O_2^- to H_2O_2 (Equation 1) Hydrogen peroxide is catalytically reduced to H_2O by catalase, a divalent oxidising enzyme (de Duve, 1978):

$$2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O \qquad (14)$$

Other mechanisms reducing intracellular H_2O_2 flux include:

a) Glutathione peroxidase (GPx) utilising a variety of intracellular electron donors:

$$RH_2 + H_2O_2 \xrightarrow{GPx} R + 2H_2O \qquad (15)$$

b) Reduced glutathione (GSH) is oxidised by H_2O_2 to oxidised glutathione (GSSG):

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O \qquad (16)$$

in consort with glutathione reductase, which regenerates GSH from GSSG. This reaction is perhaps the major intracellular H_2O_2 decomposing system. Hydrogen peroxide by reacting with metal chelates results in OH$^{\cdot}$ formation via the Fenton-type reaction (Equations 10-13).

Hydrogen peroxide is also the substrate for the myeloperoxidase reaction which generates oxidative products such as hypochlorite and possibly O_2 ($^1\Delta$ g) (Rosen and Klebanoff, 1977).

Hydrogen peroxide can exist as an anion (HO_2^-), but the pKa is high so that almost all of the molecule exists as H_2O_2 under physiologic conditions. Hydrogen peroxide is a strong oxidant, but its reactions with organic substances are slow (Fee and Valentine, 1977). Many H_2O_2-mediated reactions may be dependent on the formation of reactive oxygen intermediates initiated by homolytic cleavage of the O-O bond by transition metals (Weiss and LoBuglio, 1982).

The oxidative species so formed may injure cells and structural components of the extracellular space (Del Maestro, 1980).

2.5.2.3 Hydroxyl Radical, OH·

It is a very reactive and, therefore, very unstable oxidising species which reacts with a large variety of organic compounds. The lifetime of OH· is very short and its toxicity may be mediated through other OH· dependent compounds such as the carbonate anion radical (Michelson, 1977) and/or by the products of OH· — induced interactions with cellular components. Hydroxyl radical is generated *in vitro* by polymorphonuclear leucocytes, monocytes and macrophages (Babior, 1978a) suggesting that it may be generated extracellularly at sites of inflammation.

The Haber-Weiss reaction (Equation 12) and the O_2^--mediated reduction of metal chelates (Equation 3 and 4) have been suggested as mechanisms for OH· generation from O_2^- (Beauchamp and Fridovich, 1970; Del Maestro, 1980). Since O_2^- and H_2O_2 are relatively non-toxic and mechanisms exist for their scavenging, an assessment of the generation and role of OH· in living systems appears essential to the understanding of tissue damage initiated by oxygen-derived free radicals (Del Maestro, 1980).

2.5.2.4 Singlet Oxygen, $O_2(^1\Delta g)$.

The outer orbitals of the oxygen molecule possess two unpaired electrons and is a triplet in its ground state ($^3\Sigma g-$) since the electron spins are parallel or unpaired. One state of oxygen molecule in which both electrons occupy the same orbital and electron spins are paired, is in the

single state ($^1\Delta g$) in which it is known as singlet oxygen, O_2 ($^1\Delta g$). By definition, O_2 ($^1\Delta g$), is not a free radical but may be considered a related species because it is very reactive and some radical reactions result in its generation. A second excited singlet state also exists and in this state the electron spins are also paired ($^1\Delta g$). The oxygen molecule (O_2) generated as a result of spontaneous dismutation, Haber-Weiss reaction, and as a result of hypochlorite generated from myeloperoxidase reaction with H_2O_2 is $O_2(^1\Delta g)$ (Khan, 1970; Kellogg and Fridovich, 1977; Rosen and Klebanoff, 1977). The generation of $O_2(^1\Delta g)$ during these reactions has been based on the concept of selective scavenging of $O_2(^1\Delta g)$ by various compounds and the stimulation of $O_2(^1\Delta g)$ dependent oxidations in aqueous medium by deuterium (Del Maestro, 1980).

2.5.2.5 Hydrogen Peroxide-Peroxidase - Halide Derived Oxidants

Neutrophils and monocytes contain a haeme-enzyme called myeloperoxidase in the primary lysosomal granules (Klebanoff, 1980; Roos, 1980). On exposure to a variety of stimuli, these cells discharge granules containing myeloperoxidase into the phagocytic vacuole or into extracellular milieu. In the presence of H_2O_2, myeloperoxidase catalyses the oxidation of halides-chloride (Cl^-), iodide (I^-) or bromide (Br^-) by transferring two electrons from the halide (the electron donor) to generate an oxidised halide:

$$H_2O_2 + X \xrightarrow{\text{Myeloperoxidase}} HOX + H_2O \qquad (17)$$

where X is Cl, I or Br. Eosinophils also contain a granule-associated peroxidase that functions similarly to myeloperoxidase (Weiss and Lubuglio, 1982).

The hypohalous acids, HOCl, HOI, and HOBr, are potent oxidants and can react with a wide variety of biologically important molecules. For example, HOCl reacts rapidly with amines, amino acids, sulphydryl compounds, thioethers, aromatics, and other unsaturated carbon groups (Held and Hurst, 1978). Hypochlorous acid may also react with other oxygen metabolites generated by phagocytes to produce new reactive intermediates. Of potential interest is the ability of O_2^- to react with HOCl to form an extremely powerful oxidant, the hydroxyl radical (HO$^\cdot$) (Long and Bielski, 1980):

$$O_2^- + HOCl \longrightarrow OH^\cdot + Cl + O_2 \qquad (18)$$

2.5.3. Role of Leucocytes in Acute Myocardial Ischaemia-Reperfusion Injury.

Leucocytes have been incriminated as playing a crucial role in the development of acute myocardial infarction. Studies on pig infarcts induced by ligating the left anterior descending coronary artery for 40 minutes followed by two hours of reperfusion revealed leucocytes greater in number in the infarcts compared to non-infarcted myocardium (Rahamathulla and Ashraf, 1984). These observations and recent reports in the literature incriminating leucocytes in

the production of cytotoxic free radicals have suggested that leucocytes may play a significant role in the causation of cellular injury in anoxia or ischaemia of the heart.

Shlafer et al. (1982a) subjected isolated, perfused rabbit hearts to two hours of global ischaemia at 27°C. The presence of superoxide dismutase and catalase in the perfusion fluid greatly enhanced left ventricular functional recovery upon reperfusion. The enzyme-treated hearts developed better left ventricular pressure, left ventricular compliance, spontaneous heart rate, coronary flow, and calculated coronary vascular resistance than did untreated controls. In these studies, the respiratory function of isolated mitochondria and the release of lactate dehydrogenase were not affected by treatment with superoxide dismutase and catalase.

In a second study, Shlafer et al. (1982b) employed parabiotically perfused, isolated cat hearts, which were subjected to the same ischaemic insult as in the former study. One group simply underwent hypothermia for five minutes before ischaemia and for five minutes after ischaemic period. A second group received a crystalloid cardioplegic solution. A third group received the cardioplegic solution supplemented with superoxide dismutase and catalase. The best return of ventricular pressure development, maximal left ventricular stroke work, rate-pressure products, and coronary blood flow were seen in heart that received the cardioplegic solutions plus enzyme treatment. Shlafer et al. (1982b), therefore, indicated

that ischaemic myocardial injury may involve cytotoxicity from oxygen-derived free radicals and metabolites such as superoxide anion, hydrogen peroxide, or both, and that this component of damage can be reduced by enzyme supplements such as superoxide dismutase or catalase.

Stewart et al (1982) studied hypothermic global ischaemia in a canine model. A group of dogs undergoing hyperkalaemic cardioplegia, with and without the addition of superoxide dismutase and mannitol, were compared with a group undergoing simple hypothermia. After 60 minutes of ischaemia and 45 minutes of reperfusion, all indicators of left ventricular function were significantly better in the enzyme-treated hearts. This group also had higher calcium uptake by the sarcoplasmic reticulum.

A Langendorff perfused rabbit heart model has been studied by Casale et al. (1983). Hearts were subjected to 3.5 hours of ischaemia at 20°C. Again, left ventricular function was appreciably protected by the administration of superoxide dismutase and catalase. Protection was evident as long as the enzymes were present at the time that reperfusion began; their presence during ischaemia provided no additional degree of protection. This study, which included light — and electron-microscoscopical cardiac examination, reported substantial qualitative preservation of cellular and subcellular morphology in treated heart.

Romson et al. (1983) demonstrated that neutrophil depletion limits infarct size and reduces the area at risk

following 90 minutes of coronary artery ligation. They postulated that this may be due to an inhibition of oxygen-derived free radical generation by neutrophils in the inflammatory response to myocardial infarction.

Hess et al. (1981) and Rowe et al. (1983) demonstrated that activated neutrophils were able to disrupt canine cardiac sarcoplasmic reticulum function via a free radical mechanism and suggested that this mechanism might play an important role in acute myocardial failure.

Rowe et al. (1984) showed that oxygen-derived free radicals and/or their metabolites generated from neutrophils activated by phorbol myristate acetate (PMA) were able to significantly disrupt cardiovascular function. The complete inhibition of the effects of PMA infusion by SOD and catalase indicated an inhibition of a free radical mechanism. The source of free radical generation was suggested to be the circulating neutrophils. This was demonstrated by protection from PMA challenge afforded by administered rabbit anti-canine neutrophil antibody, which also resulted in the partial depletion of circulating neutrophils.

Anti-canine leucocyte antiserum was administered to dogs, which resulted in 77 $\pm$ 2 percent reduction in circulating neutrophils. Acute myocardial infarction was then induced, followed by six hours of reperfusion. The anti serum-treated group demonstrated a 43 percent reduction in infarct size compared to non-immune-serum-injected animals. Also histopathologic examination revealed that

infarcted myocardium from the central group demonstrated substantial neutrophilic infiltration that was virtually absent in the anti-serum-treated group (Romson et al. 1983).

In animal models with permanent coronary artery occlusion, leucocytes migrated to regions of myocardial injury within 24 hours, reaching a peak in four to five days (Mallory et al., 1939). Sommers and Jennings (1964) reported that reinstituting myocardial blood flow after temporary coronary occlusion accelerated the infiltration of inflammatory cells into injured myocardium. Romson et al. (1982, 1983), using a model of myocardial ischaemia-reperfusion injury in dogs resulting from a 60-minute occlusion of the circumflex coronary artery followed by reperfusion, observed leucocyte accumulation in the infarcted myocardium 24 hours later.

Neutrophil-generated oxygen-derived free radicals and metabolites have been shown to contribute to the tissue injury associated with acute myocardial infarction. In myocardial ischaemia-reperfusion injury in dogs, it was observed that neutrophil depletion resulted in myocardial infarcts that were approximately 40 percent smaller than infarcts in control animals with no neutrophil depletion (Romson et al., 1983). In addition, dogs treated with free radical-scavenging agents such as SOD, catalase, or mannitol, had reduced injury to the myocardium and increased recovery of left ventricular function following reperfusion (Gardner et al., 1983).

Since neutrophil depletion limits myocardial infarct size in canine model of ischaemia-reperfusion injury, the effects of ibuprofen pretreatment on inhibition of infarct size were studied by Hess et al. (1985). Their studies indicated that myocardial infarct size, as a percentage of area at risk, was reduced in the ibuprofen-pretreated group.

The effects of ibuprofen pretreatment on the canine heart subjected to temporary coronary artery occlusion followed by 24 hours of reperfusion markedly reduced the leucocyte accumulation ratio: only 76 percent increase was noted compared to 397 percent for myocardial section obtained from non-treated control animals (Hess et al., 1985).

The ability of ibuprofen, an inhibitor of free radical generation from the leucocytes, to inhibit myocardial ischaemia-reperfusion injury has also been correlated with its ability to block the activation of circulating granulocytes and inhibit their recruitment into injured myocardial sites (Romson et al., 1982; Gardner et al., 1983; Flynn et al., 1984). Thus, the mechanism underlying myocardial destruction may be based on the production of oxygen-derived free radicals. It is conceivable that this pathophysiologic cascade may open up new avenues for the diagnosis of myocardial ischaemia - reperfusion injury as well as potential application of scavengers and/or inhibitors of oxygen-derived free radicals as another potential mechanism in myocardial protection during ischaemia-reperfusion injury.

2.6 The Role of Phorbol Myristate Acetate as a Potent Leucocyte Stimulator

The influence of phorbol myristate acetate (PMA), the active principle of croton oil, on the structure and function of leucocytes have been investigated by Repine et al. (1974), DeChatelet et al. (1976), Shoyab and Todaro (1980), Weiss (1980) and Lehren and Cohen (1981). PMA has diverse effects on cellular growth, differentiation and metabolism. It is also a strong inflammatory agent (Hecker and Smith, 1974) which induces superoxide production in neutrophils (Repine et al., 1974; DeChatelet et al., 1976) through activation of NADPH oxidase (Suzuki and Lehren, 1980):

$$2O_2 + NADPH \xrightarrow[\text{PMA}]{\text{NADPH}} 2O_2^- + NADP^+ + H^+ \qquad (19)$$

Driedger and Blumberg (1980) reported that specific, high affinity receptors for phorbol diesters, such as PMA exist in chick embryo fibroblasts. Shoyab and Todaro (1980) extended that observation to include a variety of normal and transformed avian and mammalian cells. In an independent pursuit of the interactions between phorbol diester and human leucocytes, Lehren and Cohen (1981) developed alternative methods to study PMA binding to cells and reported that the rate and extent of occupancy of these receptors govern the activation of neutrophils' respiratory burst after exposure to PMA.

The addition of PMA in microgram quantities to a suspension of resting human polymorphonuclear leucocytes (PMNL) resulted in increased oxygen consumption, hexose monophosphate shunt activity, and reduction of nitroblue tetrazolium dye, similar to that observed when the cells were challenged with phagocytisable particles such as heat-killed bacteria (Repine et al., 1974). PMA also caused ultrastructural changes in cells resembling those observed during phagocytosis (White and Estensen, 1974). Defective cells that were not capable of responding metabolically to a phagocytic stimulus such as cells obtained from patients with chronic granulomatous disease of childhood (Holmes et al., 1967) showed no alterations in oxidative metabolism when exposed to PMA (Repine et al., 1974). These observations indicated that PMA enhances the generation of free radicals and metabolites by leucocytes. Thus, PMA may serve as an important component of a model system for investigating biochemical alterations during phagocytosis.

The addition of 0.1µg/ml of PMA to a suspension of resting human neutrophils caused a marked stimulation of all aspects of cellular oxidative metabolism normally associated with phagocytosis. PMA induced a greatly increased rate of glucose oxidation via the hexose monophosphate shunt and increased production of superoxide anion. The time course of hexose chemiluminescence were similar to those observed following phagocytosis of opsonised zymosan; the levels of activation achieved in all cases approximated those seen

following phagocytosis (DeChatelet et al., 1976).

Human neutrophils stimulated with PMA are capable of mediating erythrocyte (RBC) cytolysis. Target cell destruction was directly related to the number of PMNs and PMA concentration employed. PMA caused a marked stimulation of all parameters of leucocyte oxidative metabolism normally associated with phagocytosis (DeChatelet et al., 1976; Weiss et al., 1977; 1978). Thus, PMA stimulated PMN hexose monophosphate shunt activity, chemiluminescence, protein iodination, and the generation of O_2^-, H_2O_2 and $OH^\cdot$ (DeChatelet et al., 1976; Weiss et al., 1978). It has been used extensively as a potent soluble stimulus of leucocyte oxidative metabolism (DeChatelet et al., 1976; Weiss et al., 1978; Nathan et al., 1979a; 1979b).

The destruction of erythrocyte targets by PMA-stimulated neutrophils is completely inhibited by SOD or catalase indicating that haemolysis requires both O_2^- and H_2O_2. Thus, neutrophils stimulated with PMA generate O_2^- and H_2O_2 which play an integral role in a novel cytotoxic mechanism (Weiss and LoBuglio, 1980).

2.7 Oxygen Derived Free-Radicals and Leucocyte Target Cell Interactions.

The biologic reactivity of leucocyte-generated oxygen-derived free radicals has been examined and in a number of simple model systems wherein leucocytes are incubated with target cells in the presence of an appropriate stimulus. Various substances have been used to stimulate leucocytes in an undirected manner such that all adjacent targets are

exposed to the released toxins. These non-specific reactions are triggered by a variety of stimuli including opsonised zymosan particles, latex spherules, plant lectins, the croton oil derivative phorbol myristate acetate (PMA), the activated complement component C5a, and endotoxin (Henney, 1977).

Leucocyte incubated with either particulate or soluble stimuli will discharge their lysosomal contents and generate oxygen-derived free radicals. If a target cell lies adjacent to the stimulated leucocyte, the target cell will non-specifically be exposed to all of those species that can diffuse from the leucocytes. With the use of stimuli, cytotoxic potential of leucocytes' armamentarium has been examined. Target cells thus tested included erythrocytes (Dallegri et al., 1983; Vercellotti et al., 1985), lymphocytes (Kraut and Sagone, 1980), phagocytes (Baehner et al., 1977), endothelial cells (Sachs et al., 1978; Weiss et al., 1981) and tumour cells (Nathan et al., 1979a; 1979b).

2.7.1 Superoxide

Simchowitz and Schur (1976a, 1976b) first demonstrated that neutrophils incubated with lectins, phytohaemagglutinin (PHA) or concanavalin (Con A), rapidly destroy chicken erythrocyte targets. In their system cytolysis required cell-cell contact and was correlated with agglutination, but did not involve erythrophagocytosis; it was dependent on anaerobic glycolysis and independent of oxidative phosphorylation. Based on the ability of lectin to

stimulate phagocyte to generate oxygen metabolites, a potential role for O_2^-, and/or H_2O_2 in cytolysis was postulated.

Further evidence for O_2^- in leucocyte-mediated cytolysis was provided by a series of studies by Weiss (1980) and Weiss and LoBuglio (1980). Human neutrophils were stimulated with PMA and examined for their ability to lyse erythrocyte targets. Neutrophils were able to mediate a powerful cytotoxic effect, and under optimal conditions, each neutrophil could destroy more than ten erythrocyte targets. Other studies on mechanism of haemolysis revealed that superoxide dismutase inhibited haemolysis. Since the only role of superoxide dismutase is to catalyse the dismutation reaction of superoxide anion to hydrogen peroxide (McCord and Fridovich, 1969), its ability to almost completely protect erythrocytes indicated that O_2^- played an important role in cytolysis (Weiss, 1980).

Since all the data indicated that O_2^- was responsible for cytotoxicity, a cooperative interaction between O_2^- and oxyhaemoglobin was considered a potential pre-requisite for haemolysis. Superoxide (O_2^-) can react with oxyhaemoglobin to form methaemoglobin and H_2O_2 (Lynch et al., 1976; Sutton et al., 1976) which can then combine to form a peroxide-methaemoglobin complex with a strong oxidising potential. Thus, the following reaction sequence is proposed to occur within the erythrocyte target:

$$O_2^- + \text{Hb-Fe}^{2+}....O_2 \xrightarrow{2H^+} \text{Hb-Fe}^{3+} + H_2O_2 + O_2 \quad (20)$$

(Oxyhaemoglobin) (Methaemoglobin)

$$H_2O_2 + \text{Hb-Fe}^{3+} \longrightarrow \text{Hb-Fe}^{3+}.....H_2O_2 \longrightarrow \text{Lysis} \quad (21)$$

(Peroxidative complex).

This scheme was supported by:

1. inability of PMA —— stimulated neutrophils to destroy erythrocytes after the oxyhaemoglobin has been derivatised to carbon monoxyhaemoglobin.
2. ability of neutrophils to destroy methaemoglobin-rich erythrocytes by a mechanism independent of O_2^- but dependent on H_2O_2.
3. marked sensitivity of methaemoglobin-rich erythrocytes to haemolysis by H_2O_2 (Weiss, 1980).
4. ability of O_2^- to traverse erythrocyte plasma membrane via the anion channels to oxidise *intracellular haemoglobin to methaemoglobin* (Lynch and Fridovich, 1978; Weiss, 1982).

2.7.2 Hydrogen peroxide, H_2O_2

Baehner et al. (1971) demonstrated the role of oxygen metabolites in phagocyte-target cell interactions by describing the ability of human polymorphonuclear leucocyte to alter glutathione levels in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes. Normal erythrocytes are armed with two enzyme systems capable of degrading H_2O_2, catalase and glutathione peroxidase-

reductase system. Catalase can directly degrade H_2O_2, which is both the electron donor and acceptor, whereas glutathione peroxidase catalyses the reduction of H_2O_2 by reduced glutathione (GSH, α- glutamylcysteinylglycine) (Equations 14-16).

The oxidised glutathione (GSSG) is again reduced to GSH by reduced nicotinamide dinucleotide phosphate (NADPH) generated from the hexose monophosphate shunt and catalysed by glutathione reductase:

$$GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{Glutathione}} 2GSH + NADP^+ \qquad (22)$$

Finally, NADPH is regenerated by the first two enzymatic steps (glucose-6-phosphate dehydrogenase, G6PD, and 6-phosphogluconate dehydrogenase, 6PGD) in the hexose monophosphate shunt:

$$\text{Glucose-6-phosphate (G6P)} + NADP \xrightarrow{G6PD} \text{6-Phosphogluconate (6-PG)} + NADPH + H^+ \qquad (23)$$

$$6\text{-PG} + NADP \xrightarrow{6\text{-PGD}} \text{Ribulose-5-phosphate} + NADPH + H^+ \qquad (24)$$

Although both catalase and the GSH reductase systems are important in protecting erythrocytes from oxidant stress, the GSH system seems to preferentially reduce low concentration of H_2O_2 (Jacob et al., 1965).

In G6PD-deficient erythrocytes, insufficient NADPH is available to reduce GSSG. Patients with this disorder may

suffer from haemolytic anaemia during infection, and Baehner et al. (1971) hypothesized that H_2O_2 generated by leucocytes in the process of phagocytosis might injure G6PD-deficient erythrocytes. GSH oxidation in G6PD-deficient erythrocytes was accelerated in the presence of cyanide possibly due to increased H_2O_2 release from the leucocyte: Cyanide would inhibit myeloperoxidase and catalase, and/or a further decline in the ability of the erythrocyte to degrade H_2O_2 as well as blocking catalase in the erythrocyte. Baehner et al. (1971) concluded that H_2O_2 could oxidise GSH in G6PD-deficient erythrocytes and may alter the cell such that in vivo survival is shortened. Human neutrophils stimulated with PMA, which is a stronger stimulus for oxygen-derived-free radical generation than latex, can oxidise haemoglobin to methaemoglobin in intact, normal erythrocytes by a mechanism dependent on H_2O_2 (Winterbourn et al. 1981; Weiss, 1982). These studies further lend support to the concept that H_2O_2 can play an important role in oxidising susceptible intracellular targets.

Greene et al. (1980) having examined the role of oxygen in Con A-dependent neutrophil-mediated cytotoxicity against chicken erythrocytes, demonstrated that at effector-to-target cell ratio of 12.5:1, Con A-stimulated human neutrophils could destroy approximately 25 to 75 percent of the chicken erythrocytes. Neutrophils isolated from patients with chronic granulomatous disease (CGD) were incapable of mediating lysis, whereas myeloperoxidase-

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deficient neutrophils could destroy the erythrocyte comparable to control cells. Thus, destruction was mediated by an oxygen-dependent, myeloperoxidase-independent mechanism. Either superoxide dismutase or catalase could partially reduce haemolysis, whereas, in the presence of both enzymes, their effects were additive.

Human neutrophils incubated with PMA oxidised haemoglobin within the intact erythrocyte by a mechanism dependent on cell-cell contact but independent of phagocytosis. Spectrophotometric examination of the erythrocyte lysates revealed that the major component formed was methaemoglobin. Also, methaemoglobin formation was directly related to the neutrophil concentration and time of incubation. The addition of superoxide dismutase or catalase modestly inhibited methaemoglobin formation, while a combination of the enzymes provided the most dramatic protection. Apparently, either O_2^- or H_2O_2 alone was capable of mediating methaemoglobin formation in the intact erythrocyte (Weiss, 1982).

According to Weiss (1982), maintenance of the intraerythrocytic haemoglobin in its oxygenated state markedly inhibited methaemoglobin formation. Blockade of the anion channels in the intact erythrocyte with sulphonated stilbenes inhibited O_2^- but not H_2O_2 from oxidising intracellular haemoglobin. Weiss (1982), therefore, suggested that neutrophil-derived O_2^- and H_2O_2 can cross the erythrocyte membrane through the anion channel or diffused directly into the intracellular space and react

with oxyhaemoglobin or deoxyhaemoglobin to form a mixture of haemoglobin oxidation products within the intact cell.

In 25 experiments, 2 x 10^6 PMA-stimulated neutrophils oxidised 81.7 $\pm$ 6.9% of the haemoglobin to methaemoglobin in 10^7 erythrocytes (RBCs) during 60-min incubation (Weiss, 1982). Erythrocytes alone contained 0.8 $\pm$ 0.4 percent methaemoglobin and this level was not affected by either PMA or neutrophil alone. Stimulated neutrophils, therefore, can rapidly oxidise almost all of the intracellular haemoglobin to methaemoglobin within a 60-min period (Weiss, 1982).

Weiss and LoBuglio (1980) investigated the cytotoxic potential of leucocyte-generated oxygen-derived free radicals and metabolites toward RBC targets and incriminated both O_2^- and H_2O_2 in the final mediator of target cell destruction. The destruction of RBC targets by PMA-stimulated PMNs was almost completely inhibited by SOD or catalase, indicating that both O_2^- and H_2O_2 were involved in target cell destruction. Kellogg and Fridovich (1977) have demonstrated that oxygen metabolites generated by the acetaldehyde-xanthine oxidase system rapidly destroyed human RBCs in a glucose-free medium. Cytolysis was inhibited by SOD or catalase.

Nathan et al. (1979a, 1979b) investigated the interaction of murine peritoneal exudate granulocyte and BCG or casein activated macrophages stimulated by PMA on a variety of murine tumour cells and erythrocytes and concluded that target cell lysis was due to H_2O_2. Nathan et

al. (1979a, 1979b) demonstrated inhibition of cytolysis with catalase, while azide had no adverse effect on cytolysis of the target cells. This cytotoxic system provides novel insight into potential mechanisms of leucocyte-mediated target cell injury.

2.7.3 Hydrogen Peroxide-Peroxidase-Halide System

Oxidants generated from in vitro model systems consisting of H_2O_2, purified peroxidases and halides can both modify cell function and mediate cytotoxicity (Clark and Klebanoff, 1975; Klebanoff, 1980). Neutrophils, eosinophils, monocytes, and mast cells may utilise their peroxidases, to modulate their own function, mediate platelet or mast cell release and destroy neoplastic cells (Fantone and Ward, 1982).

During ingestion, neutrophils release myeloperoxidase into both the phagocyte vacuole and extracellular milieu. Thus, in the presence of H_2O_2 and a halide, the phagocyte is exposed to myeloperoxidase-generated oxidants at multiple sites.

The myeloperoxidase system is an important component in the termination of the respiratory burst, the inactivation of lysosomal enzymes, and autotoxicity (Weiss and LoBuglio, 1982).

A kinetic study of O_2^- production by normal and myeloperoxidase-deficient neutrophils revealed that initial rates of generation by the stimulated cells were comparable. However, O_2^- production by normal cells ceased after

approximately 60 minutes, whereas it continued for at least two hours with the myeloperoxidase-deficient neutrophils (Weiss and LoBuglio, 1982).

Jandl et al. (1978) examined the various factors involved in the O_2^- forming activity in the 27,000g particulate fraction from neutrophils exposed to zymosan. After a 60-minute incubation period, the O_2^- forming activity almost completely ceased. However, in the presence of myeloperoxidase inhibitors, O_2^--forming activity was preserved for approximately ten minutes. These results indicated that the respiratory burst was significantly prolonged in the absence of myeloperoxidase and O_2^--forming activity of intact neutrophils declined after stimulation by an early myeloperoxidase-dependent mechanism.

Enzyme release under aerobic conditions was apparently increased in the presence of catalase, azide and a number of scavengers, most of which react with HOCl (Skosey et al., 1981). HOCl can reversibly or irreversibly inactivate myeloperoxidase, suggesting that the enzyme may self-deactivate in the absence of the oxidisable substrates (Harrison and Schultz, 1976).

Tsan and Denison (1980) examined the ability of PMA-stimulated neutrophils to mediate autotoxicity. Injury was blocked by catalase and histidine or methionine, compounds known to react with HOCl. However, azide did not inhibit autotoxicity.

Weiss and LoBuglio (1982) suggested that the H_2O_2-myeloperoxidase-Cl^- system is involved in autotoxicity, but

in the presence of azide, the H_2O_2 concentration reaches toxic levels and destroys the neutrophil by a myeloperoxidase-independent mechanism.

The addition of myeloperoxidase inhibitors such as azide and cyanide to normal or myeloperoxidase — deficient neutrophils incubated with platelets and opsonised zymosan significantly enhanced the release of adenine and serotonin by a mechanism dependent on H_2O_2 and a halide (Clark and Klebanoff, 1980). These results suggested that H_2O_2 alone in the presence of haeme-enzyme inhibitors could enhance the release reaction. Either reagent or enzymatically generated H_2O_2 could initiate a release reaction that was stimulated by the addition of azide, cyanide, or aminotriazole, probably as a consequence of platelet's catalase inhibition. Thus, in several <u>in vitro</u> models, H_2O_2 alone can mediate effects similar to those observed with the intact myeloperoxidase system, but only if conditions allow high concentrations of H_2O_2 to accumulate. The ability of the peroxidase system to initiate a release reaction (Equation 17) is not limited to neutrophils and platelets.

Henderson <u>et al</u>. (1980a, 1980b) postulated that activated eosinophils might alter mast cell activity. In an <u>in vitro</u> model system, H_2O_2, eosinophil peroxidase, and a halide could either induce the specific secretion of granules from intact mast cells or mediate mast cell destruction depending on the eosinophil peroxidase concentration. Eosinophil peroxidase could also bind to the

matrix of mast cell granules in a manner that potentiated its secretory activity on mast cells. The eosinophil peroxidase has the opportunity to amplify its own activity by inducing mast cell granule exocytosis and binding to the released matrix.

Thus, myeloperoxidase, eosinophil peroxidase, and mast cell peroxidase are all members of a specialised class of peroxidases that may generate free hypohalous acids utilised to either modulate cell function, enhance or attenuate the generation of oxidants, or mediate cytotoxicity. These oxidants are self-contained and, independently acting together, capable of attacking target cells at multiple sites (Weiss and LoBuglio, 1982).

2.8 Role of Oxygen-Derived Free Radicals in Methaemoglobin Formation and Haemolysis

The potential role of cell-derived oxygen free radicals and metabolites in haemoglobin oxidation was initially investigated by examining the ability of neutrophils isolated from patients with chronic granulomatous diseases (CGD) to mediate methaemoglobin formation. Such neutrophils can discharge lysosomal enzymes, but cannot generate oxygen-derived free radicals and metabolites (Klebanoff, 1980). After incubating 2×10^6 neutrophils from CGD patient for 60 minutes with 10^7 erythrocytes in the presence of PMA, there was no evidence of methaemoglobin formation. This experiment demonstrated a critical requirement for oxygen-derived free radicals and metabolites in methaemoglobin formation.

Based on the ability of either O_2^- or H_2O_2 to react with cell-free solutions of oxyhaemoglobin to form methaemoglobin (Lynch et al., 1976; Sutton et al., 1976; Winterbourn et al., 1976; Lynch et al., 1977), the role of these species in methaemoglobin formation in neutrophil — erythrocyte system has been reported.

Weiss and LoBuglio (1982) incubated 2×10^6 neutrophils with 2×10^7 erythrocytes in the presence of PMA and found that the amount of methaemoglobin formed began to plateau after 45 minutes. Copper — zinc (Cu-Zn) superoxide dismutase or catalase alone mediated a partially protective effect; the simultaneous addition of both enzymes was very effective for inhibiting methaemoglobin formation. When either or both enzymes were added to a mixture of erythrocytes and PMA-stimulated neutrophils at the end of a 45-minute incubation period, no protective effect was observed. Quantitative determination of the ability of the enzymes to inhibit methaemoglobin formation showed that Cu-Zn superoxide dismutase consistently inhibited methaemoglobin formation, while neither heat nor H_2O_2-inactivated superoxide dismutase had any protective effect.

Increasing the superoxide dismutase concentration (100µg/ml.) did not enhance protective effect. Similar results were obtained when an equal number of units of purified iron-superoxide dismutase was used. Catalase alone had a slightly greater effect than the superoxide dismutase (Weiss and LoBuglio, 1982).

Kellogg and Fridovich (1977) demonstrated that an acetaldehyde-xanthine oxidase system was capable of lysing human erythrocyte targets. Either superoxide dismutase or catalase protected the erythrocyte from haemolysis. In addition, both $OH^{\cdot}$ and $O_2(^1\Delta g)$ scavangers inhibited cytotoxicity, while treatment of target cells had no protective effect. In an _in vitro_ model of cell-mediated cytotoxicity, Nathan _et al_. (1979b) examined the interaction between murine macrophages stimulated with PMA and murine erythrocytes and concluded that target cell lysis was due to H_2O_2.

Weiss (1980) also investigated the haemolytic role of superoxide in human neutrophils, and observed that haemolysis was prevented by conversion of haemoglobin to carbon monoxyhaemoglobin, but the conversion of haemoglobin to methaemoglobin by nitrite treatment did not impair neutrophil-mediated haemolysis. Nitrite-treated erythrocytes were more sensitive to H_2O_2 than control cells. This haemolysis was completely prevented by catalase.

Hess _et al_. (1985) investigated the ability of activated leucocytes to generate reduced oxygen intermediates capable of disrupting calcium transport in cardiac sarcoplasmic reticulum. They observed that in the presence of catalase, there was a significant inhibition of the disruptive effect of PMA-activated leucocytes. Neither boiled catalase nor boiled superoxide dismutase had any effects on PMA—activated leucocyte depression of cardiac sarcoplasmic reticulum (SR). The addition of SOD or

catalase alone resulted in a small but significant inhibition of PMA—activated leucocytes. In the presence of SOD plus catalase, the PMA-activated leucocyte system was completely inhibited. Since SOD and catalase are known scavengers of superoxide radical and hydrogen peroxide, such finding incriminated both O_2^- and H_2O_2 in the disruption or damage of the SR.

Erythrocytes contain a variety of defence mechanism designed to protect the cell from oxidant stress. The first defence against oxygen reduction is the copper-containing enzyme, superoxide dismutase formerly called erythrocuprein. Superoxide dismutase converts two molecules of superoxide into oxygen and hydrogen peroxide (Equation 1) (Fridovich, 1972). This first step in superoxide dismutation is crucial to the erythrocytes as it removes a free radical capable of initiating chain reactions. The dismutation product of hydrogen peroxide is handled by two mechanisms. Catalase causes direct breakdown of hydrogen peroxide to oxygen and water; but, probably, the more important mechanism is the reduction of hydrogen peroxide by reduced glutathione (GSH), catalysed by glutathione peroxidase (GPx) (Equation 16). GSH is likely to be involved in superoxide and hydroxyl radical reduction and certainly capable of reducing other free radicals in the erythrocyte (Kosower et al., 1969). The resulting oxidised glutathione (GSSG) is reduced in turn by NADPH from the pentose phosphate pathway (Equation 22).

The overall result is a linked metabolic pathway, in

which increased turnover of methaemoglobin will result in both increased activity of the glycolytic pathway and of the pentose phosphate shunt (Figure 1).

The relatively close package of haemoglobin within the erythrocyte makes it a prime target for reaction with oxygen-derived radicals and metabolites. It can be considered as an additional buffer to the glutathione system, in protecting sensitive components such as the cell membrane against internally released oxidants. The most reactive site in haemoglobin is the haem group itself. Both hydrogen peroxide and superoxide will react with haemoglobin to give methaemoglobin (Carrel et al., 1975).

The glutathione system and haemoglobin will act as oxidant traps within the red cell, but when they are exhausted, or by-passed, in the course of a steady production of oxidants, then membrane components are at risk. Although peroxidative damage to cell lipids may occur, protein structural components and enzymes will also be susceptible, particularly if they contain sulphydryl groups where the damage may involve splitting of essential disulphide bonds as has been inferred in induced paroxysmal nocturnal haemoglobinuria (Goldstein, 1974).

In view of these protective mechanisms contained in the erythrocytes, exposure of normal erythrocytes to O_2^- or H_2O_2 does not result in haemoglobin oxidation unless the experiments are performed in the presence of azide or the absence of glucose (Baehner et al., 1971; Kellog and Fridovich, 1977; Miura et al., 1978; Sullivan and Stern,

1980). Azide is a potent inhibitor of haem-enzymes, thus reinforcing the effects of leucocyte-generated free radicals and markedly stimulating leucocyte-mediated cytotoxicity. It could block cell catalase or inhibit mitochondrial metabolism and increase cell sensitivity to H_2O_2 (Weiss *et al.*, 1981). In the presence of azide, intracellular catalase is blocked, while under glucose-free conditions, NADPH cannot be continuously supplied to glutathione reductase for reduced glutathione generation (Equation 22) (Weiss, 1982); thus, resulting in increased susceptibility of RBCs to the attack of oxygen-derived free radicals and therefore, increased haemolysis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Animals

A total of 25 adult mongrel dogs of either sex, one to three years old, weighing 8 - 10 kg were used for the experiment. Eleven of the 25 dogs were experimentally induced with myocardial ischaemia-reperfusion injury, while four were sham-operated and served as controls. Seven others were induced with permanent myocardial ischaemia, while three dogs were sham-operated and served as controls.

Before surgery, all dogs were conditioned for 14 days during which they were screened for possible diseases and dewormed with pyrantel pamoate (10mg/kg) (Pfizer Products, Lagos).

3.2 Physical Examination

Twenty four hours pre- and post-surgical induction of myocardial ischaemia-reperfusion injury (MIRI) or sham operation, in each dog, rectal temperature was recorded by inserting the thermometer (Terumo MCH 58, U.S.A.) into the rectum via the anus. The heart rate was examined by auscultation using the stethoscope (Medtekhnika, U.S.S.R.) in the left lower third of the thorax in the fourth and fifth intercostal spaces.

The lungs were auscultated using the same stethoscope in the left thorax in the intercostal space. During auscultation of the heart and lungs, the heart and

respiratory rates of each dog were taken, respectively.

Only healthy dogs were used for surgical induction of myocardial ischaemia-reperfusion injury or sham operation.

3.3 Surgical Induction of Acute Myocardial Ischaemia-Reperfusion Injury.

Each dog was anaesthetised by intravenous injection of a three percent solution of sodium pentobarbital at 30 mg/kg body weight. Intubation was performed using sterile inflatable cuffed endotracheal tube (Portex Limited, Hythe, Kent, England), connected via rubber tube to the respirator (E & M Instrument Co. Inc. Houston, Texas, U.S.A.) [Pate I (Appendix D)].

Surgical procedures were conducted according to standard aseptic procedures. By left thoracotomy in the fifth intercostal space, access was obtained to the heart. The pericardium was incised and a branch of the left anterior descending coronary artery (LADCA) was occluded for one hour by ligation using size No. 1 chromic catgut (Plate II and III, Appendix D). After one hour, the ligature was removed, thereby inducing reperfusion injury (Stewart et al., 1982; Hess et al., 1985).

Occlusion of the left anterior descending coronary artery was performed in eleven dogs, while in four dogs only left thoracotomy and pericardial incision were done; without the occlusion of the coronary artery. These four sham-operated dogs served as control animals.

Seven dogs were thereafter experimentally induced with permanent ischaemia by occlusion of a branch of the anterior

descending coronary artery as above, but without subsequent removal of ligature (Sommers and Jennings, 1964). Three sham-operated dogs served as controls for this group.

During surgery, while the thoracic cavity was still open, respiration was sustained using the respirator (E & M Instrument Co. Houston, Texas, U.S.A.). After surgical closure of the chest, the respirator was disconnected only after normal respiration was restored. The process of surgical induction is shown in plates I-III (Appendix D).

3.4 Laboratory Analysis

3.4.1 Blood Sample Collection

Twenty-four hours pre- and post-surgery, venous blood samples were aseptically drawn from each dog with 21G x 1.5" needles and disposable polyethylene syringes into two separate citrated tubes. Leucocytes were harvested from one sample, while the other sample was used for haematologic examination. Twenty-four hours pre- and post-surgery, 15ml of venous blood sample was aseptically drawn from a goat, one to two years old, using sterile 19G x 1.5" needles and polyethylene syringes, into citrated tubes.

After collection, samples were immediately transported into the laboratory for analysis as follows:

3.4.2 Leucocyte Preparation

In the laboratory, leucocytes were harvested from canine blood samples as follows:

Each sample was transferred into a beaker, and 10ml of

0.15M of chilled (4°C) ammonium chloride (NH_4Cl) solution was added. The mixture was allowed to stand until the erythrocytes (RBCs) have been lysed as evidenced through the change in colour from dark red to laked colour.

Blood was then transferred into plastic tubes and centrifuged (Lourdes Instrument Corp.) at 500 x g for seven minutes, after which the supernatant fluid was decanted. The separated leucocyte pellets were washed with phosphate-buffered saline (PBS), pH 7.4, transferred into tubes and centrifuged (Damon/IEC Division, Needhamts, Mass., U.S.A.) at 150 x g for five minutes. The supernatant fluid was aspirated using Pasteur pipette. The washing with subsequent centrifugation were repeated four times.

Leucocyte concentration per microlitre of PBS was determined according to normal routine laboratory procedure using the method of Coles (1974).

3.4.3 Erythrocyte Preparation

Each blood sample was centrifuged (150 x g for five minutes) after which the supernatant fluid was aspirated as above.

Thereafter, the RBCs were treated with 2mM sodium azide (NaN_3) solution for five minutes. Leucocytes were washed with chilled PBS, centrifuged (150 x g for five minutes) and the supernatant fluid aspirated using Pasteur pipette. This procedure was repeated five times.

Erythrocyte concentration per microlitre was determined according to routine laboratory procedure using the method

of Schalm et al. (1975).

3.4.4 Incubation Mixture

As shown in table 1 , leucocytes harvested from each canine blood sample were divided into four groups: I, II, V, and VI. To two groups (I and V) were added 1ml of phorbol myristate acetate (PMA) - 200 ng/ml, (Sigma Chemical Co., Mo.), while PMA was not added to the other two groups (II and VI). Prepared target RBCs were then added to groups I and II. The ratio of WBC to RBC mixture was 1:10. Tubes in groups III and IV contained PMA-treated and -untreated RBC, respectively. Each assay group was run in triplicate.

Table 1 : Assay Groups and their Incubation Mixture.

Group	Contents	No. of Tubes
I	WBC + RBC + PMA	3
II	WBC + RBC	3
III	RBC + PMA	3
IV	RBC	3
V	WBC + PMA	3
VI	WBC	3

3.4.5 Leucocyte-Erythrocyte Cytotoxicity Assay Incubation

Immediately after constituting the incubation mixture, assay contact was initiated by centrifugation at 350 x g for five minutes.

After, the supernatant fluid was decanted and the

pellets thus obtained were incubated at 37°C in water bath (Gallenkamp, England) for one hour in a humidified atmosphere of air.

Post-incubation, the RBC - WBC interaction was arrested by adding 10ml. of chilled PBS into each tube, contents of tubes were mixed and then centrifuged at 150 x g for five minutes.

3.4.6. Methaemoglobin Determination

After centrifugation, the supernatant fluid was aspirated and the methaemoglobin contents measured spectrophotometrically using Spectronic 20 (Bausch and Lomb) at the wavelength of 540nm (Siddons and Mills, 1981).

3.4.7 Haematologic Examination

3.4.7.1 Total Leucocyte Count was determined using routine haemacytometre counting method described by Coles (1974).

3.4.7.2 Differential Leucocyte Count was performed using routine laboratory methods of counting lymphocytes, neutrophils, eosinophils, basophils, monocytes and band cells on Giemsa-stained thin blood smear and using the immersion oil at the magnification of x 100. A total of 100 WBCs were counted (Schalm et al., 1975).

3.5 Statistical Evaluation

Pre- and post-surgical leucocyte cytotoxicity for each dog was calculated based on the degree of haemolysis. Leucocyte cytotoxicity in the presence of PMA was calculated

by deducting the mean absorbance obtained for the supernatant fluid in test tube group III (RBC + PMA) from the mean absorbance obtained for supernatant fluid of test tube group I (WBC + RBC + PMA). Leucocyte cytotoxicity without PMA was calculated by deducting the absorbance obtained for supernatant fluid in test tube group IV (RBC) from that for test tube group II (WBC + RBC). The absorbance for supernatant fluid in groups V and VI was zero.

All data obtained were subjected to statistical analysis. Means and standard deviations were calculated and values were subjected to Student's t-test. Values of $P<0.05$ were considered significant.

CHAPTER 4

RESULTS

4.1 Rectal Temperature:

Induction of dogs with either MIRI or MI resulted in a rise in rectal temperature above the normal range ($P<0.01$) (Tables 2 and 6). Sham operation was associated with an insignificant rise in rectal temperature ($P> 0.05$) (Tables 3 and 7). Mean rectal temperature increased from 38.52 $\pm$ 0.45^{o}C to 39.15 $\pm$ 0.61^{o}C and from 38.40 $\pm$ 0.40^{o}C to 39.44 $\pm$ 0.22^{o}C in dogs induced with MIRI and MI, respectively (Tables 2, 3, 6, 7 and Appendix A). The rectal temperatures of dogs before induction were within the normal temperature range and did not differ from those obtained for control dogs before sham operation ($P> 0.05$) (Tables 4 and 8). Post-induction, the rise in rectal temperature was slight and not different from that of the sham-operated controls ($P> 0.05$) (Tables 4, 5, 8, 9 and Appendix A).

4.2 Respiratory Rate:

Either MIRI or MI resulted in tachypnoea. Mean respiratory rate of dogs induced with MIRI increased from 19.55 $\pm$ 6.09 to 30.46 $\pm$ 10.40 per minute ($P<0.01$) (Tables 2 and 6). Following MI induction, mean respiratory rate of dogs increased from 16.29 $\pm$ 3.40 to 28.14 $\pm$ 6.18 per minute ($P<0.05$) (Tables 2, 3, 6, 7 and Appendix A). Induction of MIRI or MI resulted in increased respiratory rates, but did not differ significantly from those of sham-operated controls ($P>0.05$) (Tables 4, 5, 8, 9 and Appendix A).

Table 2: Effect of myocardial ischaemia-reperfusion injury on mean rectal temperature, heart rate and respiratory rate.

Dog Group	Heart Rate/ Minute (Mean ± 1SD)	Respiratory Rate/Minute (Mean ± 1SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Pre-Induction (n = 11)	106.45 ± 22.54	19.55 ± 6.09	38.52 ± 0.45
Post-Induction (n = 11)	144.00*** ± 24.25	30.46*** ± 10.40	39.15*** ± 0.61

*** = P<0.01

Table 3: Effect of sham operation on mean rectal temperature, heart rate and respiratory rate of dogs used as control for myocardial ischaemia-reperfusion injury experiment.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Pre-Sham Operation (No Surgery) (n = 3)	109.00 ± 21.07	20.25 ± 5.91	38.4 ± 0.27
Post-Sham-Operation (Surgery - Induction) (n = 3)	122.00* ± 26.90	27.25* ± 12.26	38.75* ± 0.33

* = P>0.05

Table 4: Mean rectal temperature, heart rate and respiratory rate of dogs before induction with myocardial ischaemia-reperfusion injury (MIRI) compared to sham-operated controls.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Pre-MIRI Induction (n = 11)	106.45 ± 22.54	19.55 ± 6.09	38.52 ± 0.45
Pre-Sham Operation (n = 4)	109.00* ± 21.07	20.25* ± 5.91	38.40* ± 0.27

* = P>0.05

Table 5: Mean rectal temperature, heart rate and respiratory rate of dogs induced with myocardial ischaemia-reperfusion injury (MIRI) compared to sham-operated controls.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Post-MIRI Induction (Surgery + Induction) (n = 11)	144.00 ± 24.25	30.46 ± 10.40	39.15 ± 0.61
Post-Sham-Operation (Surgery-Induction) (n = 4)	122.00* ± 26.90	27.25* ± 12.60	38.75* ± 0.33

* = P>0.05

Table 6: Effect of myocardial ischaemia (MI) on mean rectal temperature, heart rate and respiratory rate.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Pre-MI Induction (n = 7)	91.43 ± 18.76	16.29 ± 3.40	38.40 ± 0.40
Post-MI Induction (n = 7)	137.29** ± 44.07	28.14** ± 6.18	39.44*** ± 0.22

** = P<0.05

*** = P<0.01

Table 7: Effect of sham operation on mean rectal temperature, heart rate and respiratory rate of dogs used as control for myocardial ischaemia experiment.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Pre-Sham Operation (No Surgery) (n = 3)	104.00 ± 21.17	19.00 ± 3.61	38.20 ± 0.30
Post-Sham Operation (Surgery-Induction) (n = 3)	125.00* ± 13.23	28.33* ± 7.51	39.10** ± 0.36

* = P>0.05

** = P<0.05

Table 8: Mean rectal temperature, heart rate and respiratory rate of dogs before induction with myocardial ischaemia (MI) compared to controls before sham operation.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Pre-MI Induction (n = 7)	91.43 ± 18.76	16.29 ± 3.40	38.40 ± 0.40
Pre-Sham Operation (n = 3)	104.00* ± 21.27	19.00* ± 3.61	38.20* ± 0.30

* = P>0.05

Table 9: Mean rectal temperature, heart rate and respiratory rate of dogs induced with myocardial ischaemia (MI) compared to sham-operated controls.

Dog Group	Heart Rate/ Minute (Mean ± 1 SD)	Respiratory Rate/Minute (Mean ± 1 SD)	Rectal Temperature, ^{o}C (Mean ± 1 SD)
Post-MI Induction (Surgery + Induction) (n = 7)	137.29 ± 44.07	28.14 ± 6.18	39.44 ± 0.22
Post-Sham Operation (Surgery - Induction) (n = 3)	125.13* ± 13.23	28.23* ± 7.51	39.10* ± 0.36

* = P>0.05

4.3 Heart Rate:

Sham operation was not associated with significant tachycardia (Tables 3 and 7), but MIRI and MI resulted in tachycardia (Tables 2 and 6). The mean heart rate was significantly increased from 106.45 ± 22.54 beats per minute before induction of dogs with MIRI to 144.00 ± 24.25 beats per minute post-MIRI induction ($P<0.01$) (Tables 2 and 6). The mean heart rate of dogs induced with MI was 91.43 ± 18.76 beats per minute before and 137.29 ± 44.07 beats per minute after induction ($P<0.05$) (Tables 2, 3, 6 and Appendix A). Significant tachycardia observed in dogs induced with MIRI or MI was not different from their corresponding sham-operated controls ($P>0.05$) (Tables 4, 5, 8 and 9 and Appendix A).

4.4 Total Leucocyte (WBC) Count:

Induction of dogs with MIRI or MI resulted in leucocytosis (Tables 10 and 12), but when compared to sham-operated dogs, the increase was not significant ($P>0.05$). Mean leucocyte count in dogs induced with MIRI rose from 10.81 ± 3.78 x $10^3/mm^3$ to 16.04 ± 4.66 x $10^3/mm^3$ and in dogs induced with MI from 12.29 ± 3.83 x $10^3/mm^3$ to 17.76 ± 5.37 x $10^3/mm^3$, but was unchanged in dogs used as controls for either MIRI or MI experiment (Tables 10-13 and Appendix B).

4.5 Differential Leucocyte Count:

4.5.1 Bands:

Neither, MIRI, MI nor sham operation induced any significant change in the number of band cells ($P>0.05$)

(Tables 10 - 13 and Appendix B).

Table 10: Effect of myocardial ischaemia-reperfusion injury on mean total and differential leucocyte counts.

	Pre-Induction (Mean ± 1 SD)	Post-Induction (Mean ± 1 SD)	Difference
Total Leucocyte Counts, ^{3} '000/mm^{3}	10.81 ± 3.78	16.04 ± 4.66	P<0.05
Bands	1.25 ± 1.04	3.00 ± 2.20	P>0.05
Neutrophils	66.38 ± 9.18	82.00 ± 4.60	P<0.01
Lymphocytes	25.63 ± 9.60	13.50 ± 5.13	P<0.01
Monocytes	1.625 ± 1.847	0.25 ± 0.707	P>0.05
Eosinophils	5.000 ± 2.828	1.250 ± 2.188	P<0.01
Basophils	0.00 ± 0.00	0.00 ± 0.00	P=0.05

n = 11

Table 11: Effect of sham operation on mean total and differential leucocyte counts of dogs used as control for myocardial ischaemia-reperfusion injury experiment.

	Pre-Surgery (Mean ± 1 SD)	Post-Surgery (Mean ± 1 SD)	Difference
Total Leucocyte Count, '000/mm^3	10.63 ± 5.86	10.93 ± 6.03	P>0.05
Bands	1.67 ± 1.53	1.33 ± 0.58	P>0.05
Neutrophils	71.33 ± 3.06	79.00 ± 1.00	P<0.01
Lymphocytes	25.67 ± 3.06	17.33 ± 3.06	P<0.01
Monocytes	0.33 ± 0.58	0.00 ± 0.00	P>0.05
Eosinophils	1.00 ± 1.00	2.33 ± 4.04	P>0.05
Basophils	0.00 ± 0.00	0.00 ± 0.00	P>0.05

n = 3

Table 12: Effect of myocardial ischaemia (MI) on mean total and differential leucocyte counts.

	Pre-Surgery (Mean ± 1 SD)	Post-Surgery (Mean ± 1 SD)	Difference
Total Leucocyte Count, '000/mm^3	12.29 ± 3.83	17.76 ± 5.37	P<0.05
Bands	0.714 ± 1.254	2.429 ± 1.718	P<0.05
Neutrophils	61.71 ± 9.79	80.29 ± 11.40	P<0.01
Lymphocytes	30.00 ± 8.15	14.29 ± 6.92	P<0.01
Monocytes	2.57 ± 1.40	0.86 ± 1.46	P>0.05
Eosinophils	5.00 ± 4.47	2.14 ± 4.49	P>0.05
Basophils	0.00 ± 0.00	0.00 ± 0.00	P>0.05

n = 7

Table 13: Effect of sham operation on mean total and differential leucocyte counts of dogs used as control for myocardical ischaemia experiment.

	Pre-Surgery (Mean ± 1 SD)	Post-Surgery (Mean ± 1 SD)	Difference
Total Leucocyte Count '000/mm^3	11.17 ± 2.71	12.13 ± 2.00	P>0.05
Bands	0.00 ± 0.00	1.00 ± 1.00	P>0.05
Neutrophils	69.00 ± 2.646	77.33 ± 4.16	P<0.05
Lymphocytes	26.33 ± 2.517	20.00 ± 5.00	P>0.05
Monocytes	1.00 ± 1.73	1.00 ± 1.00	P>0.05
Eosinophils	3.33 ± 2.08	4.00 ± 1.73	P>0.05
Basophils	0.00 ± 0.00	0.00 ± 0.00	P>0.05

n = 3

4.5.2 Neutrophils:

Neutrophilia was induced by MIRI and MI, but the increase in neutrophil counts was not different from that of the corresponding sham-operated controls ($P>0.05$) (Tables 10 - 13 and Appendix B).

4.5.3 Lymphocytes, Monocytes, Eosinophils and Basophils:

Induction of dogs with either MIRI or MI resulted in decrease in eosinophil ($P<0.05$) and lymphocyte ($P<0.01$) counts. Neither MIRI nor MI resulted in significant alteration in monocyte or basophil counts ($P>0.05$). Sham operation also induced similar changes in lymphocyte and eosinophil counts ($P>0.05$) (Tables 10 - 13 and Appendix B).

4.6 Leucocyte Cytotoxicity:

Both MIRI and MI induction resulted in increased leucocyte cytotoxicity as demonstrated by increased haemolysis ($P<0.01$) (Tables 14 and 18). Sham operation did not result in increased haemolysis ($P>0.05$) (Tables 15 and 19). This post-induction increase in haemolysis occurred either with or without PMA in the incubation mixture and was significantly different from those of sham-operated controls ($P<0.05$). (Tables 17 and 21 and Appendix C).

Addition of PMA to incubation mixture before MIRI or MI induction and sham operation did not induce increase in leucocyte cytotoxicity ($P>0.05$) (Tables 16 and 20 and Appendix C). But in the presence of PMA, sham operation or MIRI or MI resulted in significant increase in leucocyte cytotoxicity ($P<0.05$) (Tables 16 and 20 and Appendix C).

Table 14: Effect of myocardial ischaemia-reperfusion injury (MIRI) on mean leucocyte-induced haemolysis

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Pre-MIRI Induction (No surgery) (n = 11)	0.041602 ± 0.027592	0.04729 ± 0.03495	P>0.05
Post-MIRI Induction (Surgery + Induction) n = 11	0.06586 ± 0.03047	0.1247 ± 0.05328	P<0.01
Difference	P<0.01	P<0.01	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

Table 15: Effect of sham operation on mean haemolysis induced by leucocytes harvested from dogs used as control for myocardial ischaemia-reperfusion injury (MIRI) experiment.

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Pre-Sham Operation (No Surgery) (n = 4)	0.0292168 ± 0.014549	0.0455675 ± 0.0223681	P>0.05
Post-Sham Operation (Surgery - MIRI Induction) (n = 4)	0.030499 ± 0.0087143	0.0599968 ± 0.0140572	P<0.05
Difference	P>0.05	P>0.05	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

Table 16: Mean haemolysis induced by leucocytes of dogs before induction with myocardial ischaemia-reperfusion injury (MIRI) compared to controls before sham operation.

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Pre-MIRI Induction (n = 11)	0.041682 ± 0.027592	0.04729 ± 0.03495	P>0.05
Pre-Sham Operation (n = 4)	0.0292168 ± 0.014549	0.0455675 ± 0.0223681	P>0.05
Difference	P>0.05	P>0.05	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

Table 17: Mean haemolysis induced by leucocytes harvested from dogs induced with myocardial ischaemia-reperfusion injury (MIRI) compared to sham-operated controls.

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Post-MIRI Induction (Surgery + Induction) (n = 11)	0.06586 ± 0.03047	0.1247 ± 0.05328	P<0.01
Post-Sham Operation (Surgery - Induction) (n = 4)	0.030499 ± 0.0087143	0.0599968 ± 0.0140572	P<0.05
Difference	P<0.05	P<0.05	

Values are expressed in absorbance

PMA = Phorbol Myristate Acetate

Table 18: Effect of myocardial ischaemia (MI) on mean leucocyte-induced haemolysis.

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Pre-MI Induction (No Surgery) (n = 7)	0.0189053 ± 0.00861566	0.0250977 ± 0.008950	P>0.05
Post-MI Induction (Surgery + Induction) (n = 7)	0.0451342 ± 0.0197368	0.1146667 ± 0.0458972	P<0.05
Difference	P<0.01	P<0.01	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

Table 19: Effect of sham operation on mean haemolysis induced by leucocytes of dogs used as control for myocardial ischaemia (MI) experiment.

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Pre-Sham Operation (No Surgery) (n = 3)	0.01550971 ± 0.00300141	0.017110 ± 0.0038921	P>0.05
Post-Sham Operation (Surgery - MI induction) (n = 3)	0.0169568 ± 0.00263173	0.0479997 ± 0.0158989	P<0.05
Difference	P>0.05	P>0.05	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

Table 20: Mean haemolysis induced by leucocytes of dogs before induction with myocardial ischaemia compared to controls before sham operation.

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Pre-MI Induction (No Surgery) (n = 7)	0.0189053 ± 0.00861566	0.0250977 ± 0.008950	P>0.05
Pre-Sham Operation (No Surgery) (n = 3)	.0.01550971 ± 0.00300141	0.017110 ± 0:0038921	P>0.05
Difference	P>0.05	P<0.05	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

Table 21: Mean haemolysis induced by leucocytes of dogs induced with myocardial ischaemia (MI) compared to sham-operated controls

Dog Group	Without PMA (Mean ± 1 SD)	With PMA (Mean ± 1 SD)	Difference
Post-MI Induction (Surgery + Induction) (n = 7)	0.0451342 ± 0.0197568	0.1146667 ± 0.0458972	P<0.01
Post-Sham Operation (Surgery - Induction) (n = 3)	0.0169568 ± 0.00263173	0.0479997 ± 0.0158989	P<0.05
Difference	P<0.05	P<0.05	

Values are expressed in absorbance

PMA = Phorbol myristate acetate

CHAPTER 5

DISCUSSION

5.1 Rectal Temperature

Elevated body temperature is a frequent finding in myocardial infarction. Temperature values following induction of myocardial infarction were above normal range in the dog. (Sokolow and McIlroy, 1977; Ishimova, 1980). The slight rise in rectal temperature observed was due to the effect of surgery. Products of necrotised or dying myocardium activate leucocytes which in turn release endogenous pyrogens. These pyrogens are transported through the systemic circulation into the thermoregulatory centres in the hypothalamus, where they decrease the sensitivity of these centres to heat stimuli, but increase. their sensitivity to cold stimuli (Yerzina, 1980; Keele et al., 1982).

Thermoreceptors sensitive to heat in the preoptic nuclei of the anterior hypothalamus are selectively responsive to leucocyte endogenous pyrogens as demonstrated by their direct introduction to anterior hypothalamus or cerebrospinal fluid (Yerzina, 1980). Increased body temperature results in a decrease in cardiac output and blood pressure. It is accompanied by heightened sympathetic tone and direct stimulation of sino-atrial node. Thus, elevated body temperature results in tachycardia (Lyutinsky et al., 1985) which is one of the physiologic compensatory mechanisms in myocardial ischaemia and

reperfusion injury aimed at restoring normal cardiovascular function including coronary blood flow and, consequently, myocardial supply of oxygen and nutrients.

5.2 Respiratory Rate

Tachypnoea was observed in dogs induced with MIRI or MI. It occurs very often in cardiovascular lesion induced by hypoxaemia and hypercapnaemia (Smirnov et al., 1981). Tachypnoea is a direct result of reflex stimulation of respiratory centre by hypercapnaemia and hypoxaemia occurring following left anterior descending coronary artery occlusion.

According to Zhuravel et al. (1985), this hyperfunction of the respiratory system is a compensatory mechanism enhancing thermal loss and enriching the tissue with oxygen as well as eliminating accumulated metabolites in the blood.

5.3 Heart Rate

Tachycardia was observed in dogs induced with either MIRI or MI. This result is in agreement with the findings of Regan et al. (1970), Becker and Pitt (1971) and Jolly et al. (1984). Sustained increased heart rate occurred in all dogs with onset of occlusion.

Reflex stimulation of beta-adrenergic receptors in heart resulting in tachycardia plays an important role in compensatory cardiovascular regulation. Tachycardia by increasing cardiac output augments cardiac work load and oxygen metabolism, which enhance greater coronary blood flow and myocardial oxygen supply. It is aimed at returning mean

systolic arterial pressure and cardiac output to normal values (Jose and Stitt, 1969).

Thus, intrinsic heart rate is consistently and quantitatively related to functional myocardial changes occurring in diseased heart, particularly to measures of the overall functional myocardial capacity as haemodynamic estimates of ventricular function. Heart rate, therefore, is the final independent myocardial property governing ventricular performance.

Development of tachycardia is linked with reflex regulation of cardiovascular function. Initial decrease in force of ventricular contraction due to necrotised or dying myocardium decreases stroke volume which in turn induces stimulation of baroreceptors in aortic arch and carotid sinus; hypoxaemia and acidosis stimulating chemoreceptors in these reflexogenic zones; venous congestion and hyperaemia in venae cavae inducing Bainbridge reflex by stimulating receptors in these veins. From these zones, impulses are transmitted and summed in cardiovascular centres located in myelencephalon which regulate frequency and force of cardiac contractions. Continuous stimulation of these reflexogenic zones results in positive chronotropism.

Although tachycardia is usually a compensatory mechanism enhancing the pumping of blood, if prolonged, it may decompensate due to increasing myocardial fatigue (Savoysky, 1985). Tachycardia also increases myocardial oxygen consumption and intensifies ischaemia resulting in

complete reduction in left ventricular contractility. This depression of left ventricular function may be compensated for by ventricular dilatation which in turn results in further increased oxygen consumption that exacerbates ischaemia and depresses myocardial function further. Thus, perpetuating this vicious cycle (Maroko and Braunwald, 1973).

5.4 Total and Differential Leucocyte Counts

One of the major findings of this study is that leucocytes play an important role in acute myocardial ischaemia-reperfusion injury. The observed neutrophilia in sham-operated dogs was due to effects of surgery. Leucocyte role in acute myocardial infarction as a reaction to tissue necrosis has been well established (Sommers and Jennings, 1964; Sokolow and McIlroy, 1977; Ishimova, 1980; Ibrahim, 1988). At 12-24 hours after coronary artery occlusion, leucocytes migrate from the marginated and medullary pools and accumulate at the margin and inward of necrotic area. Leucocyte infiltration covers the entire ischaemic area, where they participate in removing the necrotic debris and allowing revascularisation as well as connective tissue formation (Engler, 1986).

It is hypothesised that at the site of insult, necrotised and dying myocytes release 'chemotactic factors which attract leucocytes, mainly neutrophils. These release lysosomal enzymes and generate oxygen-derived free radicals and metabolites involved in scavenging necrotised or dying

myocytes, they are also involved in myocardial damage in acute myocardial ischaemia-reperfusion injury (Hess et al., 1985; Ajimulayev, 1986; Marklund, 1988).

5.5 Leucocyte Cytotoxicity

5.5.1 Free Radical Production and Haemolysis

This study demonstrated the ability of leucocytes harvested from blood samples obtained from dogs with experimentally induced myocardial ischaemia-reperfusion injury to mediate significant cytolysis ($P<0.01$) against sensitised caprine erythrocyte targets within a 60-minute incubation period.

The activation of effector cells by phorbol myristate acetate (PMA) is necessary for target cell lysis since resting leucocytes do not exert any significant cytotoxic effect (Dallegri et al., 1983). Significant haemolysis demonstrated by PMA untreated leucocytes post-induction of MIRI or MI might be due to their activation by the nectrotised myocardium. The low dose of PMA (200ng/ml) used might be responsible for insignificant leucocyte cytotoxicity ($P>0.05$) occurring before surgery. Vercellotti et al. (1985) used 10µg/ml. of PMA to stimulate leucocytes to mediate significant haemolysis.

Results obtained using chronic granulomatous disease (CGD) neutrophils had been shown by Dallegri et al. (1983) that ox erythrocyte destruction by PMA-activated neutrophils required oxygen-derived free radical production. Unlike normal neutrophils, CGD neutrophils failed to exert any

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cytotoxicity.

5.5.2 Erythrocyte Protective Mechanism

Erythrocytes contain a variety of defence mechanisms designed to protect the cell from oxidant stress, including superoxide dismutase, catalase and the glutathione peroxidase-reductase system. Exposure of normal erythrocytes to O_2^- or H_2O_2 does not result in haemoglobin oxidation unless in the presence of azide, an intracellular catalase blocker, or under glucose-free conditions, when $NADPH_2$ could not be continuously supplied to glutathione reductase for glutathione generation. Increased haemolysis occurs in patients deficient in glucose-6-phosphate dehydrogenase (G6PD); an enzyme involved in conversion of glucose-6-phosphate (G6P) to 6-phosphoglucuronic acid in the first step of hexose monophosphate shunt that yields $NADPH_2$ (Equation 23). The generated $NADPH_2$ is involved in reduction of glutathione from the oxidised to reduced form (Equation 22). Reduced NADP in the presence of NADPH oxidase is involved in free radical generation (Equation 9). Superoxide radical dismutates in the presence of superoxide dismutase and two protons to generate hydrogen peroxide and oxygen (Equation 1). Reduced glutathione is involved in H_2O_2 scavenging (Equation 16).

Thus, glucose oxidation through the HMPS is involved in $NADPH_2$ production for the oxidase and H_2O_2 degradation. It is proposed that haemolytic anaemias such as occur in G6PD, reduced glutathione deficiencies and excessive production of

oxygen-derived free radicals as well as metabolites occur when antioxidant defence mechanisms of the erythrocytes are exhausted and/or overwhelmed as in the *in vitro* model system.

Weiss (1980), however, demonstrated that neutrophils can mediate methaemoglobin formation in normal erythrocytes. Dallegri *et al*. (1983) prepared erythrocyte-leucocyte assay mixture for periods ranging from 3-18 hours before significant haemolysis could be obtained using unsensitised erythrocytes. It appears, therefore, that prolonged interaction of erythrocyte with oxygen-derived free radicals and metabolites increases the degree of haemolysis. In view of the prolonged period required for haemolysis to occur, assay mixture in this study comprised leucocytes and erythrocytes pre-treated with 2mM of sodium azide, a powerful inhibitor of erythrocyte antioxidant defense mechanism, particularly catalase (Equation 14). Azide pretreatment of erythrocytes enabled statistically significant haemolysis ($P<0.05$) to occur within a 60-minute incubation period, thus reducing the duration of assay period.

5.5.3 Mechanism of Cytotoxicity of Leucocyte-Generated Free Radicals and Metabolites

One important mechanism by which oxygen-derived free radicals and metabolites exert their cytotoxic effects is their ability to react with unsaturated lipids and initiate lipid peroxidation reactions in target cell membranes (Kellogg and Fridovich, 1977). Peroxidation of polyunsaturated lipids has been shown to alter physiologic

state of cytomembranes, causing increased permeability of cell membranes (Maridonneau et al., 1983). Additional studies have shown that oxygen-derived free radicals and metabolites are capable of oxidising and reducing reactive groups such as sulphydryl groups on amino acids and proteins, thereby potentially altering the physical and biological properties of specific proteins such as $alpha_1$-antiprotease and mucopolysaccharides including hyaluronic acid (Matheson et al., 1979).

Leucocytes generate O_2^- and H_2O_2 into the extracellular environment and O_2 ($^1\Delta g$) as well as OH$^\cdot$ which are highly reactive compounds reacting with nearest target molecule. Although the cell membrane is believed to be the primary site of injury by leucocyte-generated free radicals and metabolites, O_2^- and H_2O_2 are capable of permeating target cell membranes via anion channels and diffusing through cytomembranes, respectively. Thus, O_2^-, H_2O_2, or their subsequent metabolites may initiate cell injury via reaction with intracellular molecules in target cells (Fanton and Ward, 1985).

In this model system, whereby harvested leucocytes incubated with sensitised caprine erythrocytes showed cytotoxicity, leucocyte-generated oxygen-derived free radicals and metabolites oxidised the haemoglobin within intact erythrocyte with subsequent haemolysis on cell-cell contact basis. Spectrophotometric analysis of the oxidised haemoglobins revealed that the major constituent of the

mixture was methaemoglobin (Weiss, 1982; Dallegri et al., 1983).

Oxyhaemoglobin which is usually represented as a ferrohaeme-O_2 complex is reduced by O_2^- to methaemoglobin and H_2O_2 (Equation 20).

Hydrogen peroxide in turn can reduce ferrohaeme-O_2 complex to methaemoglobin, water and oxygen:

$$H_2O_2 + Hb\text{-}Fe^{2+} \text{ ---- } O_2 \xrightarrow{2H^+} Hb\text{-}Fe^{3+} + 2H_2O + O_2 \quad (25)$$

Methaemoglobin in form of ferrihaeme-O_2 complex can then react with H_2O_2 forming a methaemoglobin-H_2O_2 complex that finally mediates the haemolysis (Equation 21) (Weiss, 1982). This forms the basis for oxidative haemolytic aneemias.

Romson et al. (1983) demonstrated that neutrophil depletion limits infarct size and reduces the area at risk following 90 minutes of coronary artery ligation. They, therefore, postulated that this was due to inhibition of oxygen derived free radical generation by neutrophils in the inflammatory response to myocardial infarction. This work demonstrated that activated leucocytes were capable of destroying sensitised caprine erythrocytes via a free radical mechanism that may play a crucial role in pathogenesis of acute myocardial ischaemia-reperfusion injury and dysfunction.

Thus, it appeared that myocardial leucocyte infiltration and free radical generation were an important component of ischaemia-reperfusion injury, and that the

mechanism underlying myocardial destruction was based on production of reduced oxygen intermediates. Further support for free radical-mediated mechanisms of myocardial ischaemia-reperfusion injury has been derived from studies demonstrating increased quantities of lipid peroxide in ischaemic myocardium. This was associated with a decrease in antioxidant protective mechanisms (Guarnieri et al., 1980).

This report further provided basic evidence that toxic oxygen species derived from invading inflammatory cells constituted a major mechanism whereby ischaemic myocardial tissue that was perfused, but otherwise viable and salvageable became irreversibly injured.

It is, therefore, proposed that drug intervention may reduce the generation of toxic oxygen species in a region of myocardial tissue that has been made ischaemic and subsequently reperfused. A potential clinical application of these observations would be the use of appropriate scavengers of oxygen radicals and metabolites, leucocyte-depleting agents, inhibitors of leucocyte respiratory burst as well as migration to site of insult. Administration of such agents may be beneficial in patients with acute coronary artery occlusion, especially if they are to undergo emergency reperfusion. Such interventions may enhance the amount of myocardium that is salvageable through blood flow restoration. If reperfusion is an important component to the overall development of ultimate infarct size, proper attention to the diagnosis of myocardial ischaemia-

reperfusion injury, manner in which oxygen is reintroduced and/or proper timing of intervention for the prevention of lipid peroxidation of cardiac myocyte membranes via reactive oxygen species could further enhance the extent to which the salvage of myocardium might be achieved.

The in vitro model system employed by co-incubating leucocytes harvested from canine blood samples with sensitised caprine erythrocytes allowed the latter to interact with generated oxygen free radicals and metabolites, a non-specific reaction known as 'leucocyte by-stander interaction'. This reaction may be of value in the development of a suitable in vitro test for detection and quantification of myocardial ischaemia-reperfusion injury. The routinely used tests are invasive and some of their other limitations are briefly as follows:

1. Serum Creatine Phosphokinase (CPK) Measurements:

   This is sensitive to variables such as CPK fraction released from necrotic tissue, body water fraction in which the enzyme is distributed and the amount of CPK cleared from plasma.

2. Radionuclide Techniques:

   The most commonly used is injection of 201 thallium or 99m technetium pyrophosphate for spotting of infarcted tissue. Although the two radionuclides give an approximation of infarcted area, they lack the resolution required to define small infarcts and

require mathematical modelling to determine configuration of the margin (Badd et al., 1979).

The in vitro model system of this work, however, served as an inexpensive, handy, non-invasive method to be used in animals. It is proposed that co-incubation of stimulated circulating leucocytes with sensitised erythrocytes will serve as a method of detection of myocardial ischaemia-reperfusion injury.

SUMMARY AND CONCLUSION

Eighteen healthy dogs were experimentally induced with acute myocardial ischaemia-reperfusion injury (MIRI) and myocardial ischaemia (MI) by occlusion of the left anterior descending branch of the coronary artery; seven sham-operated dogs served as controls. Twenty-four hours pre- and post-surgery, rectal temperature, heart rate and respiratory rate of each dog were recorded; citrated blood samples were obtained for total and differential leucocyte counts as well as leucocyte cytotoxicity assay. Leucocytes were examined for their capacity to mediate cytotoxicity against sensitised caprine erythrocytes in the presence of phorbol myristate acetate (PMA), a system widely employed as in vitro model of tissue damage by stimulated leucocytes.

Occlusion of the left anterior descending branch of coronary artery either with (MIRI) or without (MI) reperfusion resulted in elevated body temperature, tachycardia and tachypnoea. Both MIRI and MI induced neutrophilia and significant haemolysis. The observed increased body temperature, tachypnoea and tachycardia as well as neutrophilia were physiologic compensatory mechanisms in myocardial ischaemia and reperfusion injury aimed at restoring normal coronary blood flow and, consequently, myocardial oxygen and nutrient supply.

In response to acute myocardial ischaemic and/or reperfusion injury, leucocytes migrated from marginated and medullary pools to accumulate at the margin and inward of

necrotic myocardium, where they removed necrotic debris and allowed revascularisation as well as connective tissue formation. While performing this scavenging role, leucocytes generated cytotoxic oxygen-derived free radicals and metabolites which might be an additional factor contributing to myocardial ischaemic and/or reperfusion injury.

In conclusion, the in vitro model system employed in this study which established the potential role of leucocytes in oxidative haemolytic anaemias might be of value in non-invasive ante-mortem diagnosis of ischaemic and/or reperfusion injury, preventable by administering leucocyte-depleting agents, free radical scavengers and inhibitors of leucocyte respiratory bursts.

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APPENDIX A

Table 1: Effect of myocardial ischaemia-reperfusion injury on rectal temperature, heart rate and respiratory rate.

Body Parameter		Dog Number 2	3	4	7	8	10	12	13	14	15	16
Rectal Temperature (^{o}C)	$^{\ddagger}a.$	39.0	39.0	38.5	37.8	38.0	38.2	38.3	38.8	38.6	38.3	39.2
	$^{\ddagger}b.$	39.4	39.5	39.4	37.5	39.6	38.9	39.4	39.5	39.5	38.5.	39.4
Heart Rate/Min.	$^{\ddagger}a.$	80	120	115	124	120	120	120	116	120	64	72
	$^{\ddagger}b.$	130	180	152	127	140	98	165	170	162	124	136
Respiratory Rate/ Min.	$^{\ddagger}a.$	14	26	20	20	16	14	12	20	25	32	16
	$^{\ddagger}b.$	30	36	31	10	36	16	25	35	48	32	36

$^{\ddagger}a$ = Pre-induction
$^{\ddagger}b$ = Post-induction

Table 2: Effect of sham operation on rectal temperature, heart rate and respiratory rate of dogs used as control for myocardial ischaemia reperfusion injury experiment.

Body Parametre		Dog Number			
		1	9	10	17
Rectal Temperature, (^{o}C)	*a.	38.6	38.5	38.5	38.0
	*b.	39.0	39.0	38.7	38.5
Heart Rate/Minute	*a.	116	130	80	110
	*b.	123	136	84	145
Respiratory Rate/ Minute	*a.	26	24	13	18
	*b.	20	40	14	35

*a. = Pre-surgery
*b. = Post-surgery.

Table 3: Effect of myocardial ischaemia on rectal temperature, heart rate and respiratory rate.

Body Parameter		Dog Number						
		1	3	5	6	7	8	9
Rectal Temperature, ^{o}C	t_a.	38.7	38.2	38.0	38.5	39.1	38.3	38.0
	t_b.	39.7	39.0	39.5	39.5	39.5	39.5	39.4
Heart Rate/Minute	t_a.	70	112	84	115	80	74	105
	t_b.	76	152	160	174	128	82	189
Respiratory Rate/Minute	t_a.	12	20	18	15	20	12	17
	t_b.	20	36	24	24	32	26	35

t_a = Pre-induction
t_b = Post-induction

Table 4: Effect of sham operation on rectal temperature, heart and respiratory rates of dogs used as control for myocardial ischaemia experiment.

Body Parameter		Dog Number		
		4	10	11
Rectal Temperature, ^{o}C	*a.	38.5	37.9	38.2
	*b.	39.5	39.0	38.8
Heart Rate/Minute	*a.	120	80	120
	*b.	135	110	130
Respiratory Rate/Minute	*a.	20	15	22
	*b.	28	21	36

*a = Pre-surgery
*b = Post-surgery.

APPENDIX B

Table 1: Effect of myocardial ischaemia-reperfusion injury on total and differential leucocyte counts.

Dog Number		Total Leucocyte $000/mm^3$	Bands	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
7.	‡a.	5.5	2	74	16	4	3	1
	‡b.	9.9	4	86	8	2	0	0
8.	‡a.	15.6	1	72	24	0	3	0
	‡b.	18.8	7	84	9	0	0	0
10	‡a.	8.8	0	77	14	0	9	0
	‡b.	13.4	1	85	13	0	1	0
12	‡a.	9.8	3	61	33	0	3	0
	‡b.	14.2	1	80	19	0	0	0
13	‡a.	14.6	2	64	23	2	9	0
	‡b.	23.9	1	73	23	0	3	0
14	‡a.	15.1	1	49	43	0	7	0
	‡b.	20.8	5	82	13	0	0	0
15	‡a.	9.1	0	62	31	4	3	0
	‡b.	13.9	2	79	13	0	6	0
16	‡a.	8.0	1	72	21	3	3	0
	‡b.	13.4	3	87	10	0	0	0

‡a = Pre-Induction
‡b = Post-Induction.

Table 2: Effect of sham operation on total and differential leucocyte counts of dogs used as control for myocardial ischaemia-reperfusion injury experiment.

Dog Number	Total Leucocyte 000/mm^3	Bands	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
9. #a.	7.2	3	68	29	0	0	0
#b.	7.5	1	78	14	0	7	0
11 #a.	17.4	2	74	23	0	1	0
#b.	17.9	2	80	18	0	0	0
17 #a.	7.3	0	72	25	1	2	0
#b.	7.4	1	79	20	0	0	0

#a = Pre-surgery
#b = Post-surgery.

Table 3: Effect of myocardial ischaemia on total and differential leucocyte counts.

Dog Number		Total Leucocyte $'000/mm^3$	Bands	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
1	#a.	10.2	0	61	30	4	5	0
	#b.	22.6	5	73	21	1	0	0
3	#a.	10.5	0	49	42	4	5	0
	#b.	12.6	4	89	7	0	0	0
5	#a.	10.8	3	67	28	2	0	0
	#b.	14.2	2	86	12	0	0	0
6	#a.	9.6	2	60	35	1	2	0
	#b.	12.8	3	78	15	1	3	0
7	#a.	9.4	0	75	20	2	3	0
	#b.	14.6	2	92	6	0	0	0
8	#a.	19.0	0	70	20	4	6	0
	#b.	25.1	1	85	14	0	0	0
9	#a.	16.5	0	50	35	1	14	0
	#b.	22.4	0	59	25	4	12	0

#a = Pre-induction
#b = Post-induction.

Table 4: Effect of sham operation on total and differential leucocyte counts of dogs used as controls for myocardical ischaemia experiment.

Dog Number		Total Leucocyte $'000/mm^3$	Bands	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
4	‡a.	8.6	0	70	26	2	2	0
	‡b.	9.4	1	76	20	0	3	0
10	‡a.	14.0	0	71	24	2	3	0
	‡b.	15.2	2	82	15	0	1	0
11	‡a.	10.9	0	66	29	2	3	0
	‡b.	11.8	0	71	25	3	1	0

‡a = Pre-surgery
‡b = Post-surgery.

APPENDIX C

Table 1: Effect of myocardial ischaemia-reperfusion injury on leucocyte-induced haemolysis (Absorbance)

Dog No.		Pre-Induction				Post-Induction			
		WR^-	R^-	WR^+	R^+	WR^-	R^-	WR^+	R^+
2	(a)	0.354	0.292	0.398	0.301	0.155	0.092	0.222	0.119
	(b)	0.357	0.301	0.398	0.301	0.171	0.125	0.252	0.131
	(c)	0.337	0.284	0.444	0.301	0.208	0.125	0.252	0.131
Mean ± SD		0.34933± 0.01079	0.29233± 0.00851	0.41333± 0.02656	0.301± 0.000	0.178 ± 0.02719	0.114 ± 0.01905	0.242 ± 0.01732	0.127 ± 0.00693
3	(a)	0.108	0.081	0.119	0.097	0.102	0.066	0.143	0.051
	(b)	0.119	0.081	0.108	0.081	0.061	0.036	0.143	0.066
	(c)	0.187	0.102	0.137	0.097	0.114	0.061	0.155	0.061
Mean ± SD		0.138 ± 0.04279	0.088 ± 0.01212	0.12133± 0.01464	0.091667± 0.00924	0.09233± 0.0278	0.05433± 0.016073	0.147 ± 0.00693	0.05933± 0.00764
4	(a)	0.125	0.081	0.155	0.097	0.171	0.066	0.301	0.051
	(b)	0.102	0.081	0.097	0.081	0.161	0.036	0.301	0.066
	(c)	0.137	0.102	0.155	0.097	0.174	0.061	0.319	0.061
Mean ± SD		0.12133± 0.01779	0.088 ± 0.01212	0.13567± 0.03349	0.09167± 0.00924	0.16867± 0.00681	0.05433± 0.16073	0.307 ± 0.01039	0.05933± 0.00764
7	(a)	0.237	0.168	0.244	0.168	0.201	0.108	0.319	0.155
	(b)	0.237	0.155	0.237	0.168	0.174	0.108	0.328	0.161
	(c)	0.237	0.155	0.234	0.222	0.222	0.155	0.345	0.155
Mean ± SD		0.237±0. 0.00	0.15933± 0.00751	0.23833± 0.00513	0.186 ± 0.03118	0.199 ± 0.0241	0.12367± 0.02714	0.33067± 0.01320	0.157 ± 0.00346
8	(a)	0.215	0.108	0.171	0.108	0.252	0.149	0.328	0.174
	(b)	0.155	0.114	0.268	0.108	0.301	0.171	0.377	0.194
	(c)	0.174	0.102	0.168	0.097	0.252	0.174	0.310	0.187
Mean ± SD		0.18133± 0.03066	0.108 ± 0.006	0.20233± 0.05689	0.104333± 0.00635	0.26833± 0.02829	0.16467± 0.01365	0.33833± 0.03467	0.185 ± 0.01015
10	(a)	0.215	0.108	0.171	0.108	0.252	0.174	0.337	0.174
	(b)	0.155	0.114	0.222	0.108	0.301	0.171	0.337	0.194
	(c)	0.201	0.102	0.168	0.097	0.252	0.149	0.367	0.187
Mean ± SD		0.19033± 0.03139	0.108 ± 0.006	0.187 ± 0.03035	0.10433± 0.00635	0.26833± 0.02829	0.16467± 0.01365	0.347 ± 0.01732	0.185 ± 0.01015

Table 1 - Cont'd.

Dog No.		Pre-Induction				Post-Induction			
		WR^-	R^-	WR^+	R^+	WR^-	R^-	WR^+	R^+
12	(a)	0.032	0.022	0.046	0.036	0.114	0.081	0.171	0.097
	(b)	0.036	0.027	0.046	0.036	0.092	0.081	0.174	0.114
	(c)	0.022	0.022	0.041	0.027	0.155	0.081	0.194	0.102
Mean ± SD		0.03 ± 0.00721	0.02367± 0.00289	0.04433± 0.00289	0.033 ± 0.00520	0.12033± 0.03197	0.081 ± 0.00	0.17967± 0.01250	0.10433± 0.00874
13	(a)	0.119	0.097	0.125	0.108	0.108	0.097	0.155	0.102
	(b)	0.119	0.097	0.125	0.108	0.114	0.097	0.187	0.119
	(c)	0.108	0.097	0.137	0.102	0.119	0.081	0.222	0.102
Mean ± SD		0.11533± 0.00635	0.097 ± 0.00	0.129 ± 0.00566	0.106 ± 0.00346	0.11367± 0.00450	0.09167± 0.00924	0.188 ± 0.03351	0.10767± 0.00981
14	(a)	0.108	0.097	0.125	0.108	0.149	0.097	0.208	0.114
	(b)	0.114	0.097	0.125	0.108	0.131	0.097	0.143	0.119
	(c)	0.114	0.097	0.125	0.102	0.125	0.081	0.198	0.102
Mean ± SD		0.112 ± 0.00346	0.097 ± 0.000	0.125 ± 0.000	0.106 ± 0.00346	0.135 ± 0.01249	0.09167± 0.00924	0.18167± 0.03421	0.11167± 0.00874
15	(a)	0.097	0.086	0.149	0.114	0.131	0.071	0.201	0.119
	(b)	0.114	0.086	0.143	0.137	0.114	0.071	0.194	0.143
	(c)	0.097	0.051	0.149	0.125	0.174	0.086	0.208	0.119
Mean ± SD		0.10267± 0.00981	0.07433± 0.02021	0.147 ± 0.00346	0.12533± 0.01150	0.13967± 0.03092	0.076 ± 0.00866	0.201 ± 0.007	0.127 ± 0.01386
16	(a)	0.097	0.086	0.137	0.114	0.119	0.086	0.268	0.143
	(b)	0.102	0.086	0.174	0.137	0.143	0.071	0.260	0.119
	(c)	0.076	0.051	0.143	0.125	0.137	0.071	0.252	0.119
Mean ± SD		0.09167± 0.01380	0.07433± 0.02021	0.15133± 0.01986	0.12533± 0.00939	0.133 ± 0.0125	0.076 ± 0.00866	0.260 ± 0.008	0.127 ± 0.01386

WR^- = Leucocytes + erythrocytes without phorbol myristate acetate
WR^+ = Leucocytes + erythrocytes with phorbol myristate acetate
R^- = Erythrocytes without phorbal myristate acetate
R^+ = Erythrocytes with phorbol myristate acetate

Table 2: Effect of sham operation on haemolysis-induced by leucocytes dogs used as controls for myocardial ischaemia-reperfusion injury (Absorbance).

Dog No.		Pre-Sham Operation				Post-Sham Operation			
		WR^-	R^-	WR^+	R^+	WR^-	R^-	WR^+	R^+
1	(a)	0.310	0.292	0.310	0.301	0.137	0.125	0.168	0.119
	(b)	0.310	0.284	0.387	0.301	0.149	0.125	0.201	0.131
	(c)	0.310	0.301	0.387	0.301	0.149	0.125	0.201	0.131
Mean ± SD		0.310 ± 0.00	0.29233± 0.00851	0.36133± 0.04446	0.301 ± 0.00	0.145 ± 0.00693	0.125 ± 0.00	0.190 ± 0.01905	0.127 ± 0.00693
9	(a)	0.201	0.149	0.155	0.108	0.208	0.174	0.319	0.215
	(b)	0.194	0.149	0.155	0.108	0.208	0.171	0.301	0.237
	(c)	0.168	0.114	0.284	0.171	0.208	0.155	0.301	0.237
Mean ± SD		0.18766± 0.01739	0.13733± 0.02021	0.198 ± 0.07448	0.129 ± 0.03637	0.208 ± 0.00	0.167 ± 0.01021	0.307 ± 0.01039	0.229 ± 0.0127
11	(a)	0.046	0.022	0.041	0.027	0.119	0.081	0.155	0.097
	(b)	0.046	0.027	0.066	0.036	0.108	0.081	0.155	0.114
	(c)	0.046	0.022	0.066	0.036	0.108	0.081	0.143	0.102
Mean ± SD		0.046 ± 0.00	0.02367± 0.00289	0.05767± 0.01443	0.033 ± 0.00520	0.11167± 0.00635	0.081 ± 0.00	0.151 ± 0.00692	0.10433± 0.00873
17	(a)	0.108	0.092	0.161	0.155	0.155	0.125	0.155	0.102
	(b)	0.108	0.086	0.161	0.125	0.155	0.119	0.161	0.119
	(c)	0.161	0.119	0.168	0.125	0.149	0.125	0.161	0.119
Mean ± SD		0.12567± 0.0306	0.099 ± 0.01756	0.1633 ± 0.00404	0.135 ± 0.01732	0.153 ± 0.00346	0.123 ± 0.00283	0.159 ± 0.00546	0.11333± 0.00981

WR^- = Leucocytes + erythrocytes without phorbol myristate acetate
WR^+ = Leucocytes + erythrocytes with phorbol myristate acetate
R^- = Leucocytes + erythrocytes without phorbol myristate acetate
R^+ = Leucocytes + erythrocytes with phorbol myristate acetate

Table 3: Effect of myocardial ischaemia on leucocyte-medicated haemolysis (Absorbance)

Dog No.		Pre-Sham Operation				Post-Sham Operation			
		WH^-	H^-	WR^+	H^+	WR	R	WR^+	H^+
1	(a)	0.027	0.009	0.036	0.036	0.071	0.027	0.092	0.041
	(b)	0.027	0.009	0.092	0.041	0.046	0.027	0.092	0.046
	(c)	0.036	0.013	0.061	0.036	0.036	0.018	0.114	0.036
Mean ± SD		0.030 ± 0.00520	0.01033± 0.00231	0.063 ± 0.0281	0.03766± 0.00289	0.051 ± 0.01803	0.0240 ± 0.00520	0.09933± 0.01270	0.0410 ± 0.005
3	(a)	0.027	0.009	0.061	0.036	0.071	0.027	0.208	0.041
	(b)	0.027	0.009	0.061	0.041	0.051	0.027	0.208	0.046
	(c)	0.027	0.013	0.076	0.036	0.061	0.018	0.208	0.036
Mean ± SD		0.027±0.00	0.01033± 0.00231	0.066 ± 0.00866	0.03766± 0.00289	0.061 ± 0.01	0.024 ± 0.00520	0.208 ± 0.00	0.041 ± 0.005
5	(a)	0.076	0.046	0.086	0.061	0.482	0.398	0.602	0.456
	(b)	0.071	0.061	0.086	0.066	0.469	0.387	0.602	0.444
	(c)	0.066	0.061	0.086	0.076	0.469	0.377	0.602	0.444
Mean ± SD		0.071 ± 0.005	0.056 ± 0.00866	0.086 ± 0.00	0.06766± 0.00764	0.4733± 0.00751	0.38733± 0.0105	0.602 ± 0.000	0.448 ± 0,00693
6	(a)	0.071	0.046	0.097	0.076	0.432	0.398	0.602	0.444
	(b)	0.071	0.061	0.097	0.066	0.432	0.387	0.602	0.444
	(c)	0.071	0.061	0.071	0.061	0.444	0.377	0.469	0.444
Mean ± SD		0.071 ± 0.00	0.056 ± 0.00866	0.08833± 0.0150	0.06766± 0.00764	0.4360 ± 0.00693	0.3873 ± 0.1050	0.55766± 0.0768	0.444 ± 0.00
7	(a)	0.027	0.027	0.046	0.036	0.061	0.032	0.102	0.058
	(b)	0.027	0.022	0.046	0.036	0.061	0.018	0.194	0.046
	(c)	0.036	0.022	0.061	0.046	0.061	0.027	0.201	0.036
Mean ± SD		0.030 ± 0.0052	0.02366± 0.00289	0.051 ± 0.0066	0.03933± 0.00577	0.061 ± 0.00	0.02566± 0.00795	0.20333± 0.0107	0.0466 ± 0.0110
8	(a)	0.061	0.027	0.071	0.036	0.092	0.027	0.137	0.056
	(b)	0.046	0.022	0.071	0.036	0.056	0.032	0.141	0.046
	(c)	0.046	0.022	0.086	0.046	0.076	0.018	0.137	0.036
Mean ± SD		0.051 ± 0.00866	0.02366± 0.00289	0.076 ± 0.00866	0.03933± 0.00577	0.07466± 0.01804	0.02566± 0.00795	0.13833± 0.00231	0.0460 ± 0.0110
9	(a)	0.056	0.027	0.061	0.036	0.041	0.018	0.102	0.032
	(b)	0.061	0.022	0.076	0.046	0.056	0.004	0.102	0.036
	(c)	0.056	0.027	0.081	0.032	0.046	0.0222	0.092	0.046
Mean ± SD		0.05766± 0.00289	0.02533± 0.00289	0.07266± 0.01041	0.038 ± 0.00721	0.04766± 0.00764	0.014733± 0.00953	0.09866± 0.057741	0.038 ± 0.00721

WR^- = Leucocytes + erythrocytes without phorbol myristate acetate
WR^+ = Leucocytes + erythrocytes with phorbol myristate acetate
R^- = Leucocytes + erythrocytes without phorbol myristate acetate
R^+ = Leucocytes + erythrocytes with phorbol myristate acetate

Table 4: Effect of sham operation on haemolysis induced by leucocytes of dogs used as controls for myocardial ischaemia experiment (Absorbance)

		Pre-surgery				Post-Surgery			
Dog No.		WR^-	R^-	WR^+	R^+	WR^-	R^-	WR^+	R^+
4	(a)	0.071	0.046	0.076	0.066	0.409	0.398	0.509	0.456
	(b)	0.071	0.061	0.092	0.076	0.409	0.387	0.523	0.456
	(c)	0.066	0.061	0.076	0.061	0.387	0.377	0.523	0.444
Mean ± SD		0.06933± 0.00289	0.056 ± 0.0066	0.08133± 0.00924	0.0676 ± 0.00764	0.40166± 0.1270	0.38733± 0.01050	0.51833± 0.0081	0.452 ± 0.00693
10	(a)	0.041	0.027	0.061	0.032	0.032	0.018	0.071	0.032
	(b)	0.032	0.0222	0.061	0.036	0.027	0.0222	0.076	0.036
	(c)	0.046	0.027	0.041	0.046	0.036	0.004	0.081	0.046
Mean ± SD		0.03966± 0.00709	0.0254 ± 0.00277	0.05433± 0.0115	0.038 ± 0.00721	0.03166± 0.00451	0.014733± 0.00953	0.0760 ± 0.005	0.038 ± 0.00721
11	(a)	0.041	0.027	0.061	0.032	0.036	0.004	0.071	0.032
	(b)	0.041	0.0222	0.066	0.036	0.036	0.018	0.076	0.046
	(c)	0.051	0.027	0.051	0.046	0.036	0.0222	0.086	0.036
Mean ± SD		0.0443 ± 0.00577	0.0254 ± 0.00277	0.05933± 0.00764	0.038 ± 0.007211	0.3433 ± 0.002887	0.014733± 0.00953	0.0776 ± 0.00764	0.038 ± 0.00721

WR^- = Leucocytes + erythrocytes without phorbol myristate.
WR^+ = Leucocytes + erythrocytes with phorbol myristate
R^+ = Leucocytes + erythrocytes without phorbol myristate
R^- = Leucocytes + erythrocytes with phorbol myristate.

APPENDIX D



PLATE I: Left thoracotomy

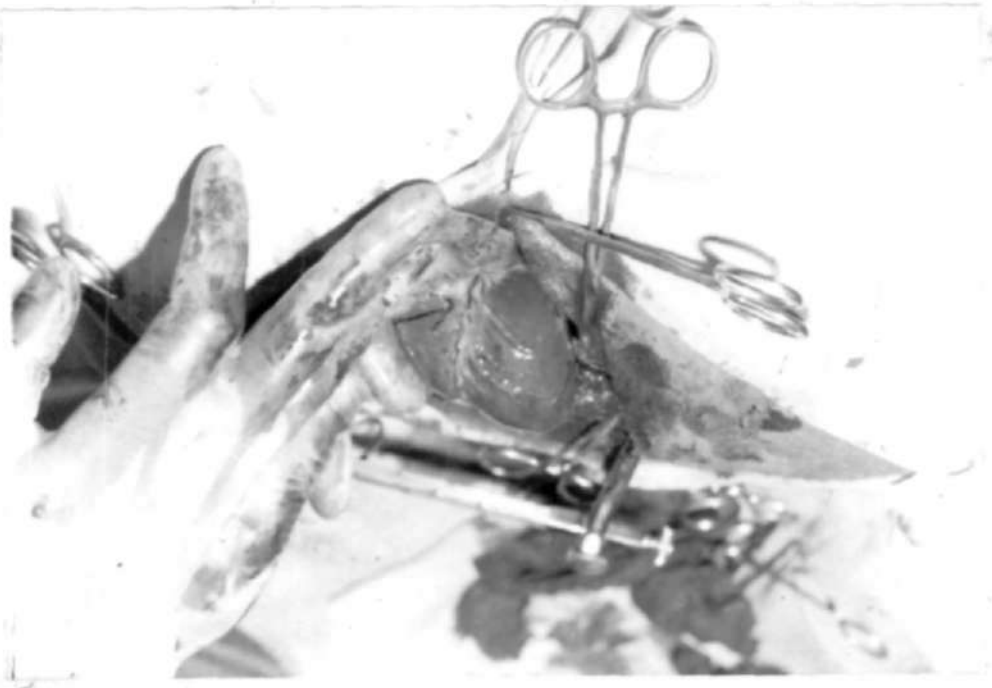


PLATE II: Left anterior descending branch of the coronary artery

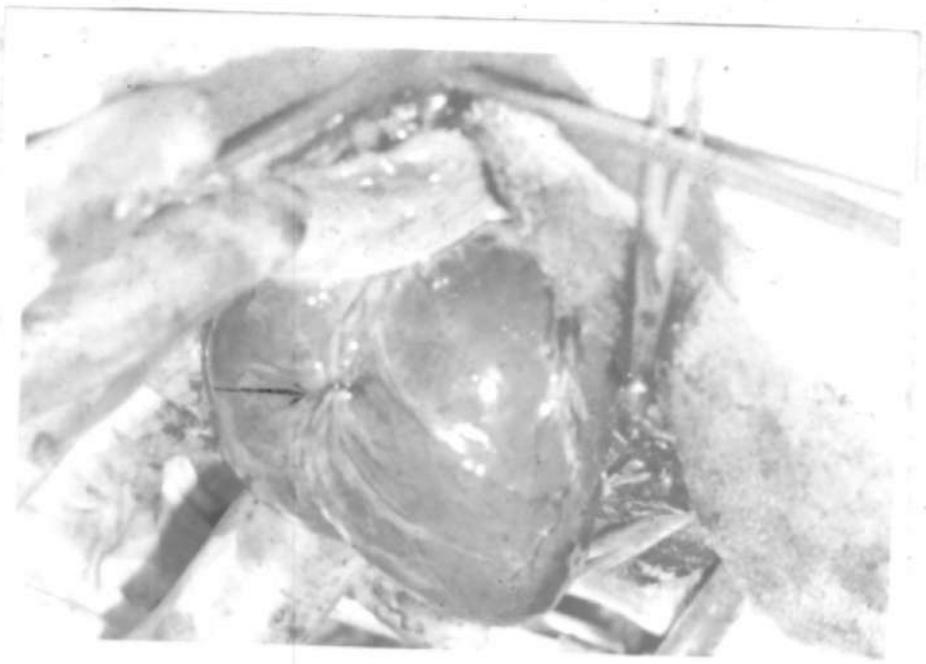
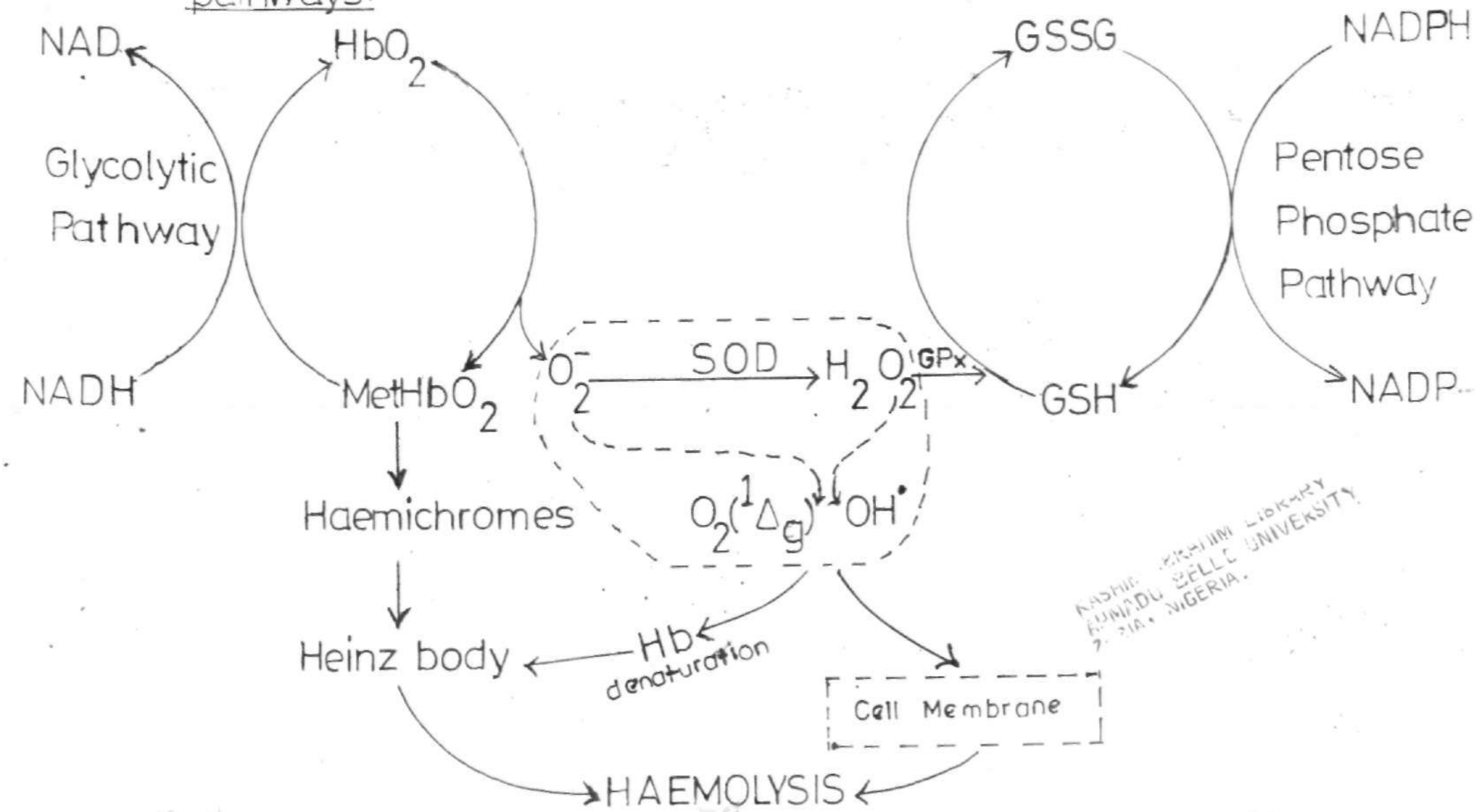


PLATE III: Occlusion of left anterior descending branch of the coronary artery

APPENDIX E

Figure 1: The generation of oxygen-derived free radicals and metabolites linked to the glycolytic and pentose phosphate pathways.



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