HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN CANINE MYOCARDIAL ISCHEMIA.

BY

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DECLARATION

I do declare that this work was carried out by me and has never been published elsewhere.

It was carried out in the Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria.

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CERTIFICATION

This thesis entitled HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN CANINE MYOCARDIAL ISCHEMIA by NAJUME DOGUWAR-GIGINYA IBRAHIM meets the regulations governing the award of the degree of Master of Science (M.Sc) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

A total of 1+2 healthy dogs with no indication of disease were used for the experiment, out of them 9 died during surgery The remaining 33 dogs were divided into 2 experimental groups.

In the 1st group of experiment the hearts of 11 dogs were infarcted by ligating LAP after thoracotomy for 1.0 hour and the ligature was removed to reperfuse the heart for 1 day; 4 dogs were control with shams incision.

In the 2nd experimental group the hearts of 16 dogs were infarcted for 1.0 hour by ligating LAD after thoracotomy and the ligature was removed to reperfuse the heart for 7 days, while 2 dogs were used as control and had shams incision. After completion of reperfusion period, dogs were euthanized and necropsy was performed on each. Grossly, the heart was rounded and flabby with scattered petecheal hemorrhages in case of reperfused hearts while control hearts were normal. Five samples were taken from the left ventricle of each reperfused and of control hearts •

Heart tissue was sectioned with minotome cryostat to obtain 5 - 6 micron thick frozen sections. Sections were histochemically stained for MPO, and counter stained with hematoxylin and eosion.

four different fields per slide were examined under light microscope and the heterogenous myocyte injury, number of leukocytes and extracellular granules were counted for each field and the data were statistically analysed.

The myocyte injury was evaluated in tissue sections and scored as 0 to 3 depending upon the structural alterations in the myrocytes, score 0 as no alteration, score 1 as mild injury, score 2 as moderate and score 3 as most severe myocyte injury.

In dogs maintained for 1 day the score 1 myocyte injury was most prevalent and the least was with score 3, while in control dogs score 0 was most common. In dogs maintained for 7 days score 2 myocyte injury was most prevalent, while the least was score 0. In the control dogs there was no myocyte injury.

There exists close relationship between heterogenous myocyte injury and the number of infiltrated leukocytes around the injured myocytes with corresponding increased released extracellular granules into the areas with heterogenous myocyte injury.

Finally, it is concluded that there is close relationship between the number of leukocytes and extracellular granules aggregation around myocytes and the intensity of myooytes injury. Leukocytes and extracellular granules release H_20_2 which is a substrate for MPO. The presence of H_2O_2 was demonstrated by using MPO specific histochemical stain and it appears that this free radical is responsible for myocyte reperfusion injury.

The implications of this investigation will be helpful for developing new drugs capable of depleting leukocytes and their extracellular granules in treating and preventing cardiac reperfusion injury. It can be used for diagnosis of death due to cardiac reperfusion injury by demonstration of MPO histochemically in frozen heart sections as MPO can not be demonstrated using other convensional staining methods. To understand the role of leukocytic H_2O_2 in the pathogenesis of reperfusion injury in the heart, which may be similar to reperfusion injury of kidney and intestine.

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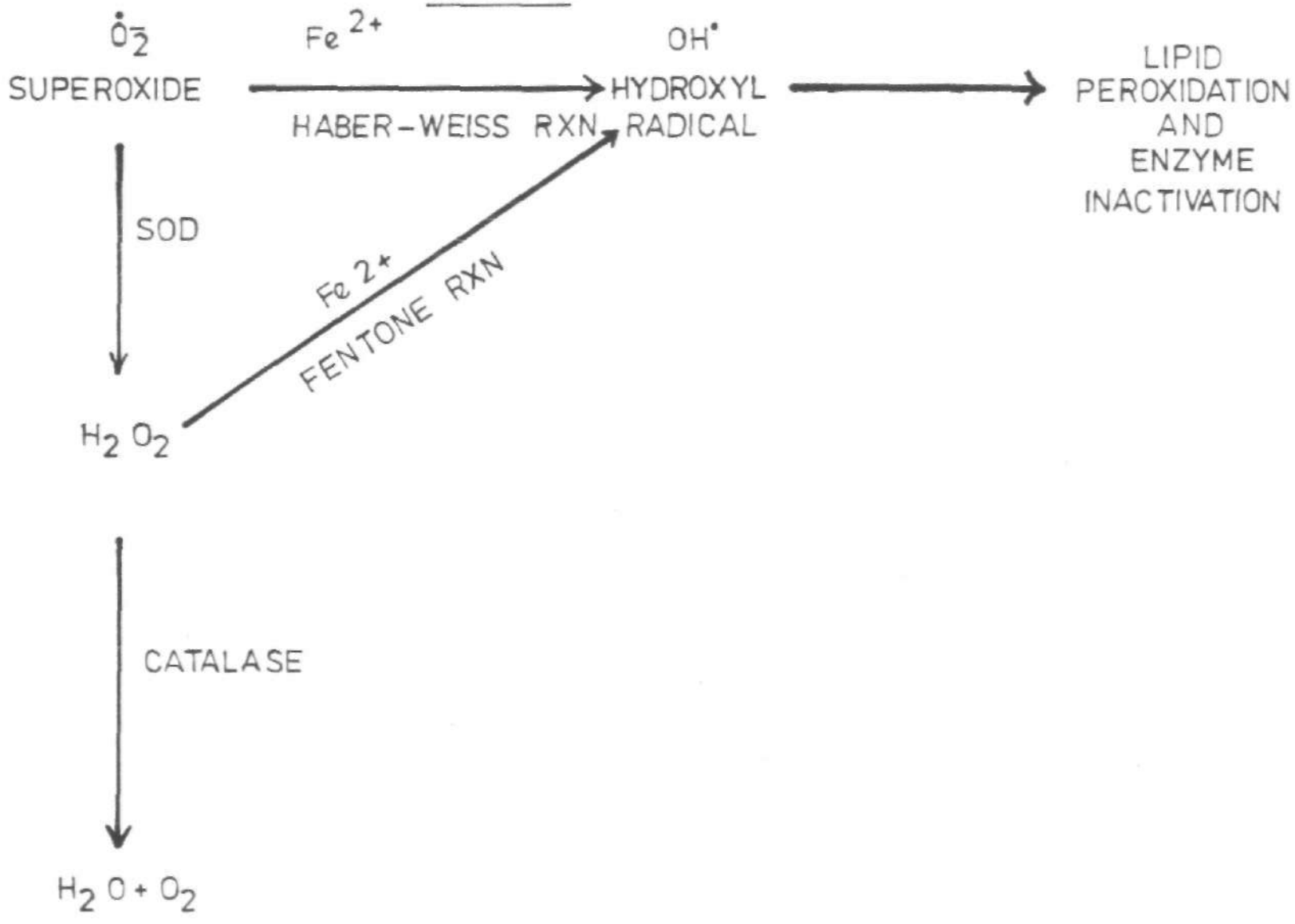
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CHART 1.



This diagram shows the role of iron reactions in the production of hydroxyl radical which is thought to be the most injurious of the oxygen based free radicals. Both superoxide and peroxide are converted through iron catalyzed reactions to hydroxyl, radical. (Myron, 1987).

CHART 2.

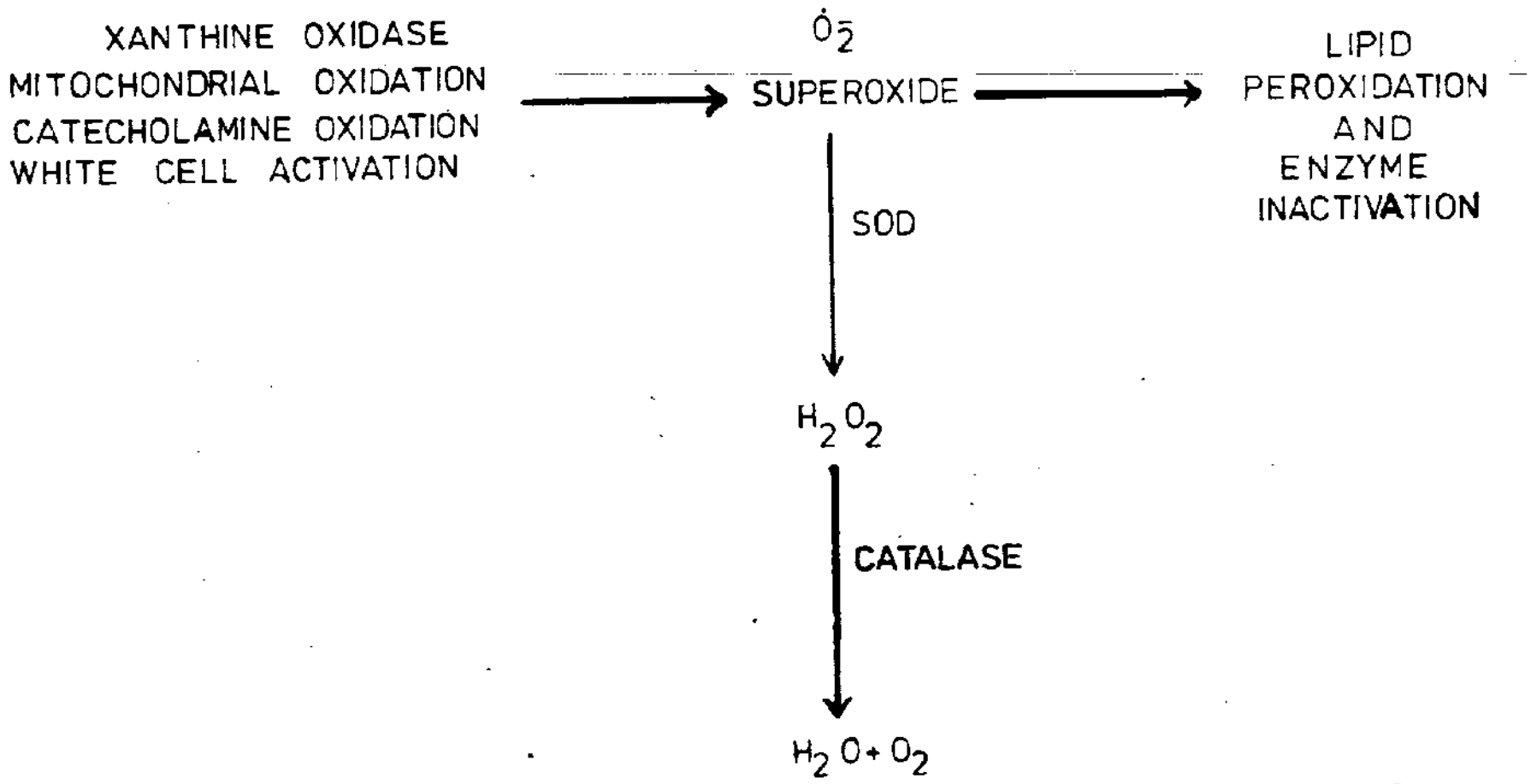


Diagram of sources of oxygen free radicals during myocardial reperfusion and intrinsic mechanisms of defense against free radical injury within myocardial cells. (Myron, 1987).

ABBREVIATIONS

i.e	That is.
LAD	Left descending branch of circumflex coronary artery.
MPO	Myeloperoxidase.

INTRODUCTION

The heart is one of the most important organs in the body. Various diseases of the heart are responsible for the frequent cases of death in animals and man. The causes of heart disease in animals and man include congenital lesions, toxins from infectious processes, bacteria, viruses, reflex vagostimulation, hypoxia, protozoa, drug induced heart block and myocardial infarction.

The incidence of heart disease is well recorded in man. In the United States, ischemic heart disease accounted for nearly two thirds of the 980,000 annual deaths of cardiovascular origin (Romson et al., 1984). Circulatory disorders account for more than half of the deaths in adults in Europe and disease of the coronary arteries is the foremost cause of deaths in males, particularly in the 40 to 60 year age group (Leathan, 1977).

Several thorough cardiovascular disease surveys have been conducted in dogs. The incidence of myocardial disease was 3.4 percent (Detweiler et al., 1968), 2.5 percent (Kerstein, 1968) and 1.3 percent (Taylor and Sittnikow, 1968), and, in each study, the incidence of myocardial disease increased with advancing age.

Myocardial infarction is one of the common conditions among the heart diseases. The usual causes of myocardial infarction are atheroma, thrombosis, arteritis, embolism, arteriosclerosis, stenosis and spasm of the blood vessels. Following infarction the body attempts to re-introduce oxygenated blood into the infarcted region of the heart, a process known as re-perfusion. This process exacerbates tissue necrosis if the duration of ischemia is more than 30 minutes. The extent of injury is proportionate to duration of ischemia. In clinical situations blood is allowed to reperfuse into ischemic region by surgical intervention such as coronary angioplasty or therapeutic agents using thrombolytic therapy. Considerable cell injury occurs

during these treatments. Various factors are implicated as the causes of myocardial cellular injury on reperfusion. These include depletion of adenosine triphosphate (ATP), excess calcium accumulation, acidosis, increased intracellular sodium, molecular oxygen and catacholamines oxidation. To counteract the harmful effects of these agents various therapeutic means, depending on aetiology, have been used. For instance to replenish ATP, a solution of glucose in saline has been infused into the heart (Hearse and Humphrey, 1975; Altschuld et al., 1981; Hohl et al., 1982). To prevent excess calcium, calcium antagonists like Verapamil, (Watts et al., 1980; Wilson, 1982), Nifedipine and Verapamil (Mayler et al., 1980) and diltiazem (Ashraf and Rahamathulla, 1984) have been used. Acidosis was prevented by using appropriate electrolyte therapy (Altschuld et al., 1981). But these proved of little value (Hess and Manson 1984). In view of this inadequacy there is a feeling that factors other than the above mentioned factors are involved in the causation of reperfusion injury.

Recently there has been a determined effort to understand the importance of oxygen free radicals in myocardial reperfusion injury. Much of this work was stimulated by the important observation of Fridovich (1978) and McCord (1985) that the xanthine oxidase system may likely generate high concentrations of free radicals at the moment of reperfusion following ischemia. Free radicals are simple molecules with odd number of electrons. They cause injury to cell membrane through chain reaction peroxidation of lipids within the membrane structure and by enzyme inactivation.

Leukocytes migrate into ischemic areas within hours of reperfusion injury initiating the acute inflammatory response. Recent reports illustrate that leukocytes are also a good source of free radicals, and hydrogen peroxide is the most important free radical. It is presumed that leukocytes generate free radicals during reperfusion and causes cell injury.

This study was conducted to test this assumption. Myocardial ischemia was produced in canine hearts by ligation of the left descending branch of the circumflex coronary artery (LAD) and reperfusion injury was induced by removing this ligation.

The hydrogen peroxide (H_2O_2) released by leukocytes is a substrate for the enzyme myeloperoxidase (MPO). H_2O_2 is involved in reperfusion injury. This was confirmed by demonstrating the presence of MPO in the intracellular and extracellular lysosomal granules of the infiltrated leukocytes, by using MPO specific histochemical stain on the frozen heart sections.

Furthermore it is also reported that all myocytes in infarcted zones are not uniformly injured and this is refered to as heterogenous cell injury. The study also considered whether infiltrated leukocytes have any role in the causation of the heterogenous cell injury in the reperfused tissue.

LITERATURE REVIEW

Myocardial infarction is an important disease in animals and humans and is often the cause of death (Charles, 1968).

Definition:-

Myocardial infarction is defined in the medical dictionary as a wedgeshaped area of dead tissue, with or without haemorrhage, produced by the obstruction of an end artery (Critchley, 1978). It is also defined as necrosis of heart muscle due to inadequate blood supply (Charles, 1968; Julian, 1978). In dog, Charles (1968) observed the infarcted area consisting of a heterogenous mixture of necrctic, living and injured myocardial tissue. The presence of viable tissue is due to the entrance of blood into parts of the infarcted area through unobstructed collateral circulation.

Myocardial infarction is also defined as necrosis of an area of myocardium, almost invariably the result of gross reduction of coronary blood flow to the affected area. Fresh coronary artery platelet thrombi are found in at least half of human post-mortem cases (Fleming and Braimbridge, 1974).

Causes of Myocardial Infarction:-

The most common cause is atheroma usually complicated by superimposed thrombosis. Other causes are arteritides, embolism, stasis following ostial stenosis, trauma, arteriosclerosis and experimental ligation of coronary vessel (Fleming and Braimbridge, 1974).

Myocardial Reperfusion Injury:-

Reperfusion injury or post-ischemic injury occurs when molecular exygen or calcium ions are reintroduced to the ischemic or hypoxic tissue by restoration of blood supply to the ischemic heart (Haglund and Lundgren, 1978).

Causes of Reperfusion Injury:-

Several perplexing results are obtained on reperfusion of previously ischemic hearts with blood or oxygenated medium.

Instead of improving cardiac function, reperfusion results in contracture, cellular necrosis and intracellular calcium overload. This phenomenon has also been termed oxygen paradox (Hearse et. al. 1973., Hearse et al., 1978; Rahamathulla and Ashraf, 1984). Similar pathology to that of the oxygen paradox has been described when calcium free perfusion of the myocardium was followed by calcium replete perfusion (Zimmerman and Hutsmann, 1966). Contracture, cellular necrosis and intracellular calcium overload develop when the calcium free perfusion medium was replaced with the calcium containing medium. The phenomenon is termed calcium paradox. Since, the oxygen paradox and calcium paradox have the same common pathology, it has been suggested that they may be "facets" of the same problem (Hearse et al., 1978). Several authors agree with this concept (Hess and Manson, 1984). In addition, it is postulated that the cardiac oxygen paradox and ischemic/ reperfusion injury share similar pathology and have common etiology i.e. reintroduction of molecular oxygen (Hess and Manson, 1984).

Currently there is a concentrated effort to understand the importance of free radicals in cardiac reperfusion injury. Equally, effort is being made to assess reduction of reperfusion injury by free radical scavengers (Myron, 1987). Much of this work has been stimulated by the important observations of McCord and Fridovich (1968), Fridovich (1978) and McCord (1985) while studying the xanthine oxidase system in which high concentrations of free radicals were generated. Free radicals cause injury to cell membrane through lipid peroxidation, enzyme inactivation and disruption of mitochondrial function resulting in cell death.

Free Radicals:-

According to McCord (1985) and Myron (1987), a free radical is a simple molecule containing an odd number of electrons or half bond, rendering it chemically reactive.

List of Free Radicals:-

Weiss and Lobuglio (1982) have listed the following free radicals

1. $\cdot O^{-}_{2}$ (Superoxide)
2. H_2O_2 (Hydrogen peroxide)
3. OH^{-} (Hydroxyl radical)
4. Hydrogen - Peroxide - Peroxidase - Halide Derived Oxidants
5. Hypochlorous acid (HOCl), Hypoiodous acid (HOI) and Hypobromous acid (HOBr).

Release of Free Radicals:-

During myocardial ischemia, free radicals are released in the following sequence:

Conditions during myocardial ischemia prime the xanthine oxidase system for immediate production of oxygen free radicals at reflow. During ischemia calcium activated proteases convert the enzyme xanthine dehydrogenase to xanthine oxidase. Hypoxanthine, a byproduct of ATP degradation is the natural substrate for xanthine oxidase. In the absence of oxygen during ischemia this reaction does not go forward; but at the moment of reperfusion with oxygen the reaction goes to completion with formation of uric acid and the oxygen free radicals, superoxide, hydroxyl radical and hydrogen peroxide (Romson et al.,1983).

Other possible mechanisms for superoxide free radical production at the moment of reperfusion include: free radical production by the electron transport chain in part as a result of injury occurring during ischemia from white cell activation within the ischemic myocardium (Romson et al., 1983 ; Engler et al., 1986),and oxidation of catecholamines at the moment of reperfusion (Myron, 1987).

Role of Leukocytes in Inflammation:-

Since the initial observations of Metchnikoff in the late 1800s, describing the presence of phagocytic cells in tissues,

a large body of information has accumulated defining the role of phagocytes in acute and chronic inflammatory reactions. When a host tissue is challenged by a pathologic insult of either an immunologic nature or pathologic agent, an inflammatory reaction may occur, with subsquent clearance of the pathologic stimulus by phagocytic cells. Tissue injury may result from either the direct effects of the pathologic agent or as a consequence of an inflammatory cell influx. Three of the most extensively studied phagocytic cells are the polymorphonuclear leukocyte (PMN), the tissue macrophage, and the circulating monocyte. Each of these cells plays a key role in acute and/or chronic inflammatory responses. When pathogenic agents are formed or deposited in host tissues, circulating neutrophils and/or monocytes may be recruited from the intravascular compartment to the site of insult by the local generation of soluble mediators. The process by which the cells migrate from the circulation to an inflammatory site is thought to involve the mechanism of direct cell migration, chemotaxis (Ward et al., 1979; Ward 1980). In vitro studies have shown that when neutrophils and monocytes are exposed to a chemotactic agent, the cell orients itself and migrates in the direction of increasing concentration of the chemical stimulus. In addition, many of the particulate and soluble phlogistic mediators generated at the sites of inflammation have been shown to activate the metabolic processes of phagocytic cells, a consequence of which is an increase in the capacity of these cells to ingest pathogenic substances (Joseph and Ward, 1982).

Source of Free Radicals:-

(a) Superoxide

After the phagocyte has been exposed to the appropriate stimulus a reduced pyridine nucleotide oxidase capable of utilizing NADH or NADPH is activated and begins to shuttle

electrons from the appropriate electron donor to oxygen (Badwey and Karnowsky 1980 ; Roos, 1980). Although the characteristics and localization of the oxidase in the leukocytes are controversial, it appears that the enzyme is membrane bound and comprises a minielectron transport chain, with a flavoprotein, a b-cytochrome, and perhaps a quinone. The reduced pyridine nucleotides can donate two electrons via the oxidase to two molecules of oxygen to form two molecules of superoxide ($O^{-}2$) (Weiss and Lobuglio, 1982).

$$2\ O_2 + NAD(P)H \xrightarrow[\text{Oxidase}]{(NA(D)PH} 2\ O_2^{\cdot -} + NA(D)PH + H^{+}$$

(b) Hydrogen Peroxide

Stoichiometric analysis of $O_2^{\cdot -}$ and H_2O_2 generation by human neutrophils ingesting opsonized bacteria revealed that about 80 per cent of the H_2O_2 generated arises from the dismutation of $O_2^{\cdot -}$ (Root and Metcalf, 1977).

Although phagocytes may generate H_2O_2 via the dismutation of $O_2^{\cdot -}$, the ability of cell's oxidase to release H_2O_2 directly remained undetermined. Certain enzymes (e.g. glucose oxidase) can apparently reduce oxygen to H_2O_2 by a two-electron pathway (Fridovich, 1976). It is conceivable that under the appropriate conditions phagocytes could generate H_2O_2 without a demonstrable $O_2^{\cdot -}$ intermediate.

(c) Hydrogen Peroxide-Peroxidase-Derived Oxidants

Neutrophils and monocytes from a variety of species contain a heme-enzyme, myeloperoxidase, in the primary lysosomal granules (Klebanoff and Clark, 1978; Roos, 1980 ; Root and Cohen 1981). Phagocytes exposed to a variety of stimuli discharge granules containing myeloperoxidase into the phagocytic vacuole or into the extracellular space. In the presence of H_2O_2, myeloperoxidase can catalyze the oxidation of the halides, like chloride (Cl^{-}), iodide (I^{-}) or bromide (Br^{-}) but not fluoride (Fl^{-}).

In this reaction, myeloperoxidase effectively transfers two electrons from the halide (the electron donor) to H_2O_2 (the electron acceptor) to generate an oxidized halide.

$$H_2O_2 + X^- \xrightarrow{\text{Myeloperoxidase}} HOX + H_2O,$$

where X is a halide = (Cl^-, I^-, Br^-).

The optimum pH for the myeloperoxidase system is approximately 4.5 to 5.0 but can be increased by both the H_2O_2 and halide concentrations (Weiss and Lobuglio, 1982).

Phagocytes ingesting particles or responding to soluble stimuli release sufficient amounts of H_2O_2 to catalyze the halogenation of susceptible target molecules. In vitro, a model system consisting of H_2O_2, purified myeloperoxidase, and Cl^- has been identified to generate a powerful oxidant hypochlorous acid (HOCl) (Harrison and Schultz, 1976). Although products of I^- and Br^- oxidations by myelo - or eosinoperoxidase have not been identified, hypoiodous (HOI) and hypobromous acid (HOBr) seem likely candidates (Weiss and Lobuglio, 1982).

(d) Hydroxyl Radical

The OH^- is an extremely powerful oxidant capable of interacting with a host of organic and inorganic molecules at diffusion controlled rates (Willson, 1979). Beauchamp and Fridovich (1970) have suggested that the two species $O_2^{\cdot -}$ and H_2O_2 interacted via the Haber-Weiss reaction to form OH^-.

$$O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2 \text{ (Haber-Weiss reaction).}$$

$OH^{\cdot}$ is a hydroxyl anion (DelMaestro et al., 1980).

Free Radical Induced Cellular Injury:-

The structural and biochemical environment along with the reactivity of the O_2 derived free radicals generated, determine the type of cellular injury sustained by a tissue.

It has been demonstrated that a large group of reducing agents degrade hyaluronic acid in vitro (Pigman et al., 1961; Herp et al., 1971), in a series of reactions referred to as oxidative - reductive depolymerization (ORD

reactions) (Pigman et al. 1961). The exposure of hyaluronic acid solutions to radiation also results in its degradation (Balazes et al., 1967). Hyaluronic acid degradation can also be induced by substrate-xanthine oxidase radical generating systems (McCord, 1974; Halliwell, 1978; Del Maestro, 1979; Del Maestro et al. 1979). The mechanism of hyaluronic acid degradation may be related to a similar mechanism resulting in the generation of OH^- (Balazes et al., 1967; Herp et al., 1971; McCord, 1974; Del Maestro et al., 1979), and this reaction is dependent on the amount and chelaxing of metal catalysts present (Del Maestro, 1979). Exposure of collagen in vitro to a substrate-xanthine oxidase system results in its structural alteration (Greenwald and Moy, 1979). The degradation of extracellur space components, such as hyaluronic acid and collagen, would alter significantly both the permeability and structural characteristics of living tissue.

Biomembranes:- The membranes of mammalian cells contain large amounts of polyunsaturated fatty acids (Rouser et al., 1968) which can undergo peroxidative injury (Mead, 1976). Lipid peroxidative chain reactions with subsequent disruption of both lysosomal and cellular membranes may be induced by enzymatically generated free radicals (Kellogg and Fridovich, 1975, 1977; Goldstein and Weissman, 1977; Thomas et al., 1978). Hydroxyl radical and possibly superoxide (Kellogg and Fridovich, 1977) may react with the unsaturated lipids of biomembranes resulting in the generation of lipid peroxide radicals ($ROO^{\cdot}$), lipid hydroperoxiles (ROOH) and fragmentation products such as malondialdehyde.

Major consequences to cellular integrity may result from the initiation of propagating radical chain reactions. If extensive regions of plasmalemmal membranes undergo peroxidative change, the products of lipid peroxidation may be released into the

extracellular space which may influence vascular permeablity (Del Maestro et al., 1980). It has also been suggested that a product of radical generation may alter phospholipase activity, which would result in arachidonic acid release and subsequent formation of stable prostaglandins and various endoperoxides (Del Maestro, 1979). Ohmori et al. (1979) have considered that histamine may be released by H_2O_2 generation by free radical reactions. Therefore, some of the vascular and permeability changesseen during inflammatory processes may be related to the products of free radical induced chain reactions on the microvasculature.

The role played by radicals in inducing intracellular biochemical and organelle damage is much less clear. In vitro experiments have demonstrated that lysosomal membranes can be disrupted by a free radical flux (Fong et al., 1973), and cells in culture develop autophagocytic vacuoles (Del Maestro, 1980) which may participate in cellular injury. DNA can also be degraded by a radical flux and this certainly plays a role in carcinogenesis as may other products of lipid peroxidation (Morgan et al.,1976). The mechanism of action of many chemotherapeutic antineoplastic agents (Fridovich, 1978) and the toxicity of compounds such as paraquat (Autor, 1974) and carbon tetrachloride (Rechnagel et al., 1977) have been linked to their ability to alter the rate of intracellular radical flux and/or the generation of new radicals. The interaction of free radicals with biomembranes and other components of the intracellular milieu may determine to a degree the type of injury sustained by a cell in certain disease processes. An essential role played by mitochondria is the detoxification of oxygen via its reduction to H_2O by the cytochrome oxidase system. This role appears to be intimately linked to the production of essential energy compounds such as ATP. The ability of mitochondria to accomplish these tasks is

dependent on the organized structural arrangement of its inner membrane (Del Maestro, 1980). Boveris (1977) has demonstrated that mitochondria generate a steady flux of $O_2^{\cdot -}$. The source of this $O_2^{\cdot -}$ is not known. However, many of the compounds localized in electron transport chain (NAD, FAD, Coenzyme Q) when reduced may react with O_2 to release $O_2^{\cdot -}$ and this may be considered the "univalent leak".

During incomplete ischemia, however, the "univalent leak" may increase and mitochondrial damage may occur depending on the length of incomplete ischemia as well as the integrity of the mitochondrial scavenging mechanisms. In the period immediately after the initiation of reperfusion, cells may be exposed to relatively large concentrations of O_2 and these cells must readjust their metabolism to deal with high O_2 tension. This relatively high O_2 tension may further damage the integrity of the mitochondria during complete or incomplete ischemic period and decrease the scavenging mechanisms which may result in a high free radical load culminating in further membrane damage and cellular death (Del Maestro, 1980).

The Action of Leukocytic Free Radicals on Myocardial Tissue:

Neutrophil infiltration of the myocardium is an important component of such diverse disease entities as myocarditis, ischemia, and ischemic reperfusion injury. Activated neutrophils are capable of disrupting myocardial function via an oxygen free-radical mechanism. Human neutrophils activated with phorbol myristate acetate disrupted calcium transport by canine cardiac sarcoplasmic reticulum. This process of disruption was inhibited by a combination of superoxide dismutase and catalase. In addition, the activated neutrophil system was also inhibited by the combination of cyclooxygenase inhibitors (ibuprofen and indomethacin) and catalase and accelerated by MK-447. These results incriminate both hydrogen peroxide and the

hydroxyl radical as mediators of neutrophil-induced myocardial dysfunction. Following 6 hours of reperfusion, there was a 43 percent reduction in infarct size compared to non-immune sera-injected animals. Oxygen free radicals generated by neutrophils are capable of inducing significant myocardial injury and play an important role in the pathophysiology of ischemia-reperfusion injury (Hess et al., 1985).

In recognition of the effect of oxygen on the extent of ischemia - induced myocardial injury, efforts to reduce experimental myocardial infarct size have been concentrated on reducing oxygen consumption or increasing oxygen delivery via collateral coronary vessels. More recently, new cardioprotective compounds have emerged that do not appear to alter myocardial oxygen supply or demand. The efficacy of these compounds have prompted reassessment of the pathologic processes in ischemic myocardial injury and has led to consideration of the role of leukocytes in myocardial infarction (Romson et al., 1984).

To assess the ability of ibuprofen, a free radical scavenger to inhibit the extent of platelet aggregation and leukocyte infiltration during acute myocardial infarction, autologous indium-111 (^{111}In) - labelled platelets or leukocytes were injected before 60 minutes of left circumflex coronary artery occlusion, followed by 24 hours of reperfusion in the canine heart. Myocardial infarct size, was reduced in the ibuprofen-treated group by 40 percent compared to control animals. Leukocyte accumulation in infarcted tissue was reduced significantly. In tissue samples with 0.41 - 0.60 gram infarct, the infarcted/normal ratio of leukocyte radioactivity was 12 ± 2 in control dogs and 4 ± 1 in ibuprofen-treated dogs, which represents a 67 percent reduction in leukocyte accumulation in ibuprofen-treated compared with control dogs. Although both platelets and leukocytes accumulate in infarcted canine myocardium, ibuprofen may exert its beneficial effect on ischemic

myocardium by suppressing the inflammatory response associated with myocardial ischemia and infarction (Romson et al., 1982).

Accumulation of neutrophils during the acute inflammatory response may exacerbate tissue injury through the release of activated oxygen products or proteolytic enzymes or both. To assess the role of neutrophils in acute myocardial infarction, circulating neutrophil levels in dogs were reduced by 77 ± 2 percent (mean ± SEM) by administering rabbit antiserum to dogs. Acute myocardial infarction was induced in these dogs by 90 minutes of left circumflex coronary artery occlusion followed by 6 hours of reperfusion. Dogs treated with neutrophil antiserum developed myocardial infarcts that were an average of 43 percent smaller than infarcts in dogs treated with non-immune rabbit serum. In a saline-treated control group infarct size was 48.0 ± 4.7 percent of the area at risk, a value not significantly different from that of the non-immune serum but significantly greater than that in the neutrophil antiserum dogs. Histopathologic examination revealed that infarcted myocardium from dogs given saline or treated with non-immune serum had a substantial neutrophilic infiltrate, which was virtually absent in infarcted tissue from dogs treated with neutrophil antiserum. These observations suggest that neutrophil accumulation in response to myocardial ischemia may be responsible for a substantial portion of the irreversible myocardial injury resulting from temporary coronary artery occlusion (Romson et al., 1983).

The effect of Nafazatrom on leukocyte function in vitro and in vivo were related to its ability to salvage ischemic myocardium in an occlusion-reperfusion model of myocardial injury in the anaesthetized dog. Nafazatrom (0.4 - 75 micro mole) produced a dose - related inhibition of neutrophil aggregation, superoxide anion generation, arachidonic acid metabolism, and to a lesser extent, the release of B-glucoronidase in vitro.

In contrast, Nafazatrom (0.4 - 37.5 micro mole) did not substantially influence platelet aggregation or the platelet metabolism of arachidonic acid. In vivo Nafazatrom (10mg/kg,) reduced infarct size from 58 ± 3 percent of the risk area (mean ± SEM) in control dogs to 23 ± 2 percent of the risk area. Nafazatrom also reduced the incidence of accompanying arrhythmias. Nafazatrom - induced myocardial salvage was not associated with any hemodynamic changes; moreover, it was independent of platelets since thrombocytopenia did not prevent Nafazatrom from exerting a protective effect. Measurement of the neutrophil-specific myeloperoxidase enzyme in ischemic myocardium indicates that the smaller infarct size in dogs treated with Nafazatrom is accompanied by diminished leukocyte infiltration. Thus, the ability of Nafazatrom to inhibit neutrophil function in vitro and cell infiltration in vivo may be responsible for its myocardial-protective effects (Bednar et al., 1985).

Recent evidence indicates that leukocytes are large, stiff, viscous cells that naturally adhere to vascular endothelium (Engler et al., 1986). Their broad role in the early myocardial microvascular response to acute ischemia was suggested by 1) the role of leukocyte capillary plugging in the no reflow phenomenon, 2) microvascular resistance increase in skeletal muscle with leukocytic infusions, and 3) salvage of ischemic myocardium by anti-leukocytic agents.

Coronary circulation was perfused under matched, controlled conditions with whole blood or granulocyte-depleted whole blood. During 1 hour of ischemia (left anterior descending occlusion) circumflex perfusion pressure was servocontrolled to a constant value. In whole blood-perfused hearts, flow measured by the radio-labelled microsphere method decreased in endocardium from 0.12 ± 0.05 at 5 minutes of ischemia to 0.09 ± 0.04 ml min^{-1} g^{-} at 60 min of ischemia and in epicardium from 0.27 ± 0.17 to 0.21 ± 0.16 ml. min^{-1}, g^{-1}, both $p<0.05$. In granulocyte-depleted

blood - perfused hearts, endocardium blood flow increased over the same period from 0.18 ± 0.15 to 0.29 ± 0.18 ml min^{-1}, g^{-1} in endocardium ($p<0.05$) and did not change significantly in epicardium (0.36 ± 0.22 to 0.41 ± 0.24 ml min^{-1}. g^{-1}). The leukocyte depleted blood perfusate contained less than 33 granulocytes/µl, whereas control perfusate contained 4,265/µl. Reperfusion at normal pressures with carbon suspension allowed for histologic evaluation of the no reflow phenomenon.

With whole blood perfusion the no-reflow phenomenon in the endocardium was present with 27 percent of capillaries occluded, compared with nearly complete reperfusion in leukocyte depleted animals (1 percent of capillaries occluded, $p<0.05$). Furthermore, leukocyte depletion prevented the increases in tissue water content seen in control hearts and decreased the incidence of ventricular arrythmias. These experiments demonstrate the significant participation of granulocytes in the unfavorable responses of flow, edema formation, and arrhythmias to the 1st hour of myocardial ischemia and further document their role in the no-reflow phenomenon (Engler et al., 1986).

Biochemical Properties of Myeloperoxidase:-

Myeloperoxidase was originally named verdoperoxidase by Agner (1941) who purified it in 1941 and was later renamed myeloperoxidase. Agner (1941) purified myeloperoxidase from human pus, crystallized it after isolation from dog empyema. The iron content is 0.074 percent, molecular weight is 149,000 and there are 2 haeme groups per molecule. Acids or heavy metals could not release the haeme group, but when the enzyme was left for 48 hours at room temperature or for 1 hour at 70°C, in 50 percent pyridine some changes occurred so that one haeme group could be precipitated by methanol and other group remained in solution. The 2 fractions showed the same absorption spectrum in 50 percent pyridine. On reduction of myeloperoxidase, the soret band moved

from 430 to 480 mu and the other band from 570 (with faint maximum at 630 and 690 mu) to 637 mu (shoulder at 590 mu). Myeloperoxidase as crystallized from ammoniacal ammonium sulfate solution, is homogenous in solubility tests, electrophoretic within p^H 4.5 to 10.5 (Ehrenberg and Agner, 1958). MPO is carbohydrate-free, is highly basic and has an isoelectric point of approximately 10.2. The amino acid composition is unknown. MPO in a series of peroxidases with purpurogallin number ranging from 10 to 500, has purpurogallin number 75 which can be obtained under the standard conditions for purpurogallin test, RZ (A soret/A protein) 0.82 (Agner, 1958; Ehrenberg and Agner,1958) and nitrogen 16.6percent (Paul et al., 1960,1963).

Neutrophils and monocytes from a variety of species contain a heme-enzyme in the primary lysosomal granules known as myeloperoxidase (Klebanoff and Clark, 1978; Klebanoff,1980; Roos, 1980; Root and Cohen, 1981). Phagocytes exposed to a variety of stimuli discharge granules containing myeloperoxidase into the phagocytic vacuole or into the extracellular milieu. In the presence of H_2O_2 ,myeloperoxidase can catalyse the oxidation of chlorides (Cl^-) iodide (I^-) or bromide (Br^-) but not fluoride (F1). In this reaction myeloperoxidase effectively transfers 2 electrons from the halide (the electron donor) to H_2O_2 (the electron acceptor) to generate an oxidized halide (Weiss and Lobuglio, 1982).

$$H_2O_2 + X \xrightarrow[(Cl^-,\ I^-,\ Br^-)]{\text{Myeloperoxidase}} HOX + H_2O,$$

where X is a halide = (Cl^-, I^-, Br^-).

The optimum p^H of the myeloperoxidase system is approximately 4.5 to 5.0 but can be regulated by both the H_2O_2 and halide concentrations. Thus, under the appropriate experimental conditions the enzyme may function over a much wider p^H range (Weiss and Lobuglio, 1982).

Inhibitors of Myeloperoxidase:-

The enzymatic activity of MPO was inhibited by cyanide, azide, fluoride and hydroxylamine, but not by carbon-monoxide (CO). Azide and fluoride gave no spectroscopically observable compound with ferric MPO, but reduction in the presence of azide gave a distinct spectrum and this is quite different from what is usually found for haemoproteins (Agner, 1941).

Lysosomal Granules and MPO:-

Ultrastractural cytochemical observations have revealed peroxidase reactivity in primary (azurophil) granules, but not in secondary granules of rabbit and human polymorphonuclear leukocytes. Peroxidase reactivity was also observed in the rough endoplasmic reticulum and granules of rabbit eosinophils and in granules of human monocytes (Dunn et al., 1968).

Staining Characteristics of Myeloperoxidase:-

The demonstration of leukocyte peroxidase activity has been of practical value to haematologists since the begining of the present century. Many techniques have been described on the use of benzidine as an indicator. This compound when oxidized precipitates as brown blue insoluble crystals or granules. In the cytoplasm of granulocytes and monocytes, peroxidase activity is represented by discrete blue granules. In eosinophils MPO stains more intensely and appears tinged brown-black or green-black granules. All other formed elements of blood and bone marrow, including basophils do not stain for MPO. Satisfactory staining of tissue granulocytes can be obtained using fresh frozen sections (Kaplow, 1965).

Hereditary Deficiency of Myeloperoxidase:-

Hereditary MPO deficiency has been described as an autosomal recessive leukocyte disorder, associated with the defective candidicidal activity in leukocytes and apparently with enhanced susceptibility to systemic candida infection.

In genetically determined leukocyte myeloperoxidase deficiency, either the enzyme is completely lacking or it is structurally altered in its enzymatic, haeme-binding and antigenic sites (Sydney et al., 1970).

Lippi et al., (1984) studied myeloperoxidase and eosinophil peroxidase activity in healthy subjects and in 4 totally myeloperoxidase deficient subjects using cytochemical and biochemical methods.

Other clinical findings (Lippi et al., 1983) indicated that myeloperoxidase deficiency, perhaps the commonest of all identifiable polymorphonuclear neutrophil defects, rarely alters host susceptibility to severe infections since alternative systems to the MPO-H_2O_2-Cl^- system exist.

Sources of Error in Demonstrating MPO:- These include:

1) Inaccurate measurement of volumes or weighing of reagents and chemicals.
2) Omission of H_2O_2 or benzidine
3) Use of expired chemical reagents
4) Inaccurate p^H and temperature
5) Improper fixing and incubation of material
6) Use of specimen that is not fresh (Kaplow, 1965).

Histopathological Features of Myocardial Injuries:-

Proportion of necrotic and living myocardial fibers vary from animal to animal (Jennings et al., 1957).

Bouchary and Manjo (1974) observed cell heterogeneity in human infarcts. They termed non-necrotic myofibrils in infarcts as wavy fibers. The histopathology of human myocardial infarcts was reviewed in a series of 46 cases ranging from sudden death to

a clinical age of 3 days. A set of histopathologic features was described whereby the diagnosis of acute myocardial infarction could be made, even in cases of sudden death, on routine sections and even after considerable autolysis. This was primarily based on a stretching and waviness of the myocardial fibers, especially at the border of the infarcted area. The mechanism of stretching and waviness of the myocardial fibers is probably 2 folds: the rhythymical pull exerted by the normal myocardium against the infarcted paralysed area and the outward buldging of this area at every systole. On the basis of human material alone, it may be inferred that this pattern develops very rapidly; surely less than 1 hour and perhaps a few minutes after the local circulation has failed.

There are hints of the presence of wavy myofibrils in the work of Mallory et al.,(1939) on myocardial infarction, especially when they studied 96-hours infarct. Sommers and Jennings (1964) reported excellent wavy fibers in their publication, and commented on the stretching but didn't mention the waviness. Waves also appeared in a figure published by Jorgensen et al. (1967) showing a 4-hour experimental infarct, and in the text book illustrations such as those of Hudson (1965), Hort (1965) and Gould (1968).

Heterogeneous cell injury and increased tissue calcium levels were observed by Rahamathulla and Ashraf (1984) in the isolated rat hearts perfused with anoxic medium for 5, 10, 15, 20, 25 and 30 minutes. These changes were intensified after reoxygenation of the anoxic hearts. The cell injury was quantitated using a score of 0, 1, 2 or 3 (0 being normal and 3 being severe injury). All 4 classes of cells were present even after 5 minutes of anoxia. Reduction in the percentage of injured cells was observed on reoxygenation of hearts perfused with anoxic medium for 5 to 20 minutes. However, reoxygenation of hearts initially perfused with anoxic medium for 25 to 30 minutes resulted in a higher percentage of injured myocytes. Beneficial effects of reoxygenation were observed in the anoxic hearts when the percentage of cells with an injury score of 1 and glycogen deposit were high, coupled with low calcium levels.

Rahamathulla et al.(1985) had again shown heterogenous cell injury in the heart after perfusion with Krebs-Henseleit medium for 24 hours. They demonstrated this in their experiment on prevention of lactate production and myocyte injury in isolated rat hearts perfused, with perfluorochemical emulsion.

The objective of this work is to study myocardial infarction in vivo and post ischemic reperfusion injury using dogs as experimental model with a view to understanding the role of leukocytes and myeloperoxidase (MPO) with relationship to degree of injury to myocytes.. This is aimed at providing information on how heart attack being a very important cause of death in animals and humans can be diagnosed from heart section at post-mortem and possible prevention.

MATERIALS AND METHODS

Source of Experimental Animals:-

Dogs used in the experiments were obtained from the Health Office, Sabon-Gari District, Zaria. The dogs were rested for 3 days, and on the fourth day blood, urine and feces were examined to detect presence of any disease. They were screened using electrocardiogram (F.J.C. - 7110 Fukuda Denshi) to eliminate presence of pre-existing cardiac diseases. The dogs were given tick-bath with 10 percent solution of Chlorfenvinphose (Pfizona(R) Pfizer products Ltd, Ikeja, Nigeria) and dewormed with Pyrantel-pamoate (Combantrin(R), Pfizer Products Ltd, Ikeja). Their temperature, pulse and respiratory rates were taken.

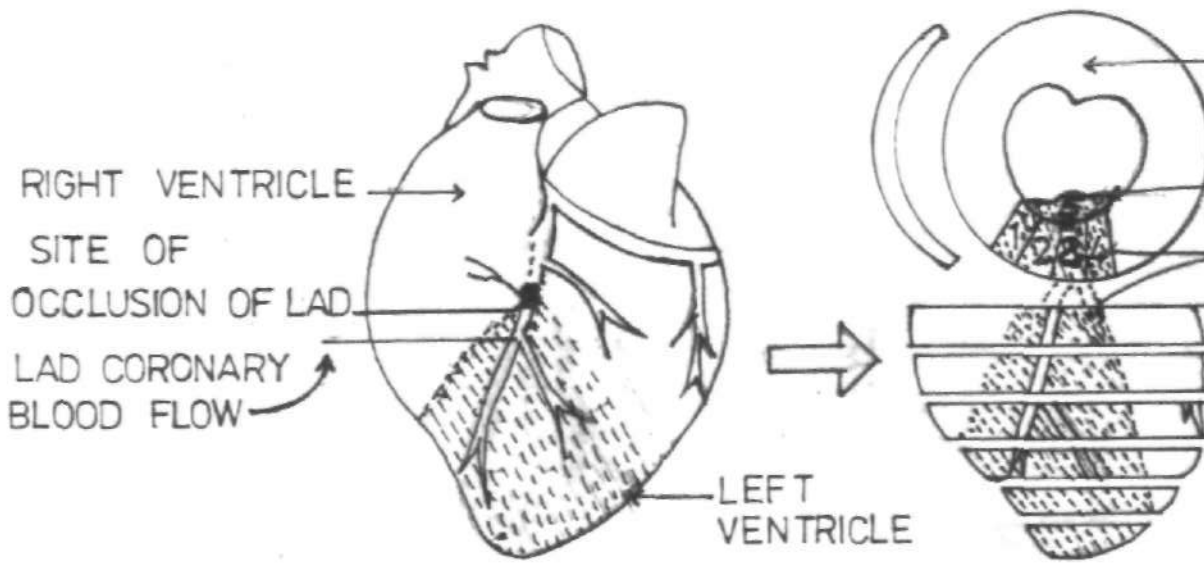
Experimental Design:

A total of 42 healthy dogs with no indication of disease were used. Out of the 42 dogs used, 9 of the dogs died during surgery. The remaining 33 dogs were divided into 2 experimental groups.

In the first experiment the hearts of 11 dogs were infarcted for 1.0 hour and reperfused for 1 day. Four dogs were used as control with shams incision and their pericardium was incised. Five random samples were taken from the heart of each of the 11 dogs from the reperfused areas at risk of the left ventricle. Five random samples were also taken from the left ventricle of each control dog (Chart 3).

The 2nd experimental group consisted of 18 dogs; the hearts of 16 of these dogs were infarcted for 1.0 hour and reperfused for 7 days. The remaining 2 dogs were used as control with shams incision and the pericardium of their hearts was incised. Five random samples were taken from reperfused areas of the left ventricle of the 16 infarcted dogs (page 29). Five random samples were also taken from the left ventricle of each control dog (Chart 3).

CHART 3.



Schemetic diagram indicating the method for producing myocardial infarction in anaesthetized dogs. The LAD coronary artery is occluded below the first major diagnol branch. At the end of 1·0 hour the ligature was removed to reperfuse the infarcted zone. The heart was removed and samples taken from different infarcted zones.

The samples were used for determination of histochemical alterations and histopathology. All the dogs were fed with rice, meat, vegetable oil and salt throughout the experiments, and water was supplied ad-libitum.

Preparation of the Animals for Surgery:-

The experimental dogs were clipped on the left lateral thorax from the scapula to the 13th rib extending from the spinal vertebrae dorsally and to the sternum ventrally. The dogs were premedicated with 1 percent atropine, and anesthesia was induced to effect with sodium pentobarbitone Nembutal(R) (Ceva Limited 3 Rhodes Way, Wartford, UK; 37 Avenue V-75008 Paris, France) at 26.43 mg/kg body weight injected intravenously. All dogs were routinely intubated with endotrachael tube for artificial respiration. The surgical site was aseptically prepared with Chlorhexidine Scrub solution (Nolvasan(R), Fort Dodge Labs. Ind., Fort Dodge, Iowa 50501), and finally 70 percent alcohol was applied.

The Surgical Procedure :-

The dogs were aseptically draped with either linen or stick on drapes (Barrier(R), Fohusm and Fuhuson Co. New Brunswick, New Jersey). A curvelinear skin incision was made at the 4th intercostal space from the dorsum to a point midway between the costochondral junction and the sternum (Ellison, 1980).

Myotomies of cutaneous trunci, lattissimus dorsi, serratus and scaleneous muscles were done to reach the rectus abdominus and intercostal muscles. Hemostasis was obtained by ligation of vessels. The incision was extended through the rectus abdominus and intercostal muscles to reach the thorax. Exposure of the thorax was increased with the use of rib retractors.

The dogs were connected to a respirator (E and M Instrument Co. INC., Houston, Texas, U.S.A.) immediately on opening the thorax, the respiratory rate was regulated at 10 to 20 per minute with an inspiratory ratio of 1:3.

The collapsed lungs were retracted to exteriorize the heart. The pericardium was incised and the branch of left descending circumflex coronary artery (LAD) of the left ventricle as shown in (Chart 3) was isolated and targeted for ligation. The LAD, was ligated with 1/0 chromic catgut for one hour and released to reperfuse the myocardium. The thorax was then closed, negative pleural pressure was achieved by maximally inflating the lungs prior to the last stitch of rib approximation followed by aspiration after final skin closure. The respirator was removed after rib closure and spontaneous breathing was allowed to resume. Extubation was done following the return of swallowing reflexes. The dogs were transfered to a clean disinfected kennel. The same surgical procedure was followed in the control dogs except that ligation of (LAD) was not done.

Post-Surgical Care:-

The temperature, pulse and respiration rates of the dogs were monitored daily. In all the operated dogs the temperature range was within 38.2°C to 38.8°C, pulse rate between 120 to 140 per minute and respiration, 20 to 28 per minute, and these parameters were within the normal range.

Necropsy Procedure:-

After 1 day in the 1st group and 7 days in the 2nd group, according to the experimental schedule, the dogs were euthanized and necropsied.

Collection of Materials:-

The thoracic cavity was opened, the heart was exteriorized and removed. The gross pathological lesions were noted and samples were taken from the infarcted zone (area at risk), Samples were taken at the same points in the control dogs (Chart 3).

The samples were immediately cut to a size of 0.5 by 0.5 cm and were fixed in 10 percent buffered formalin (pH 7.4) for 2 - 3 minutes.

Sectioning Procedure:-

The heart tissues were placed on metal discs in minotome cryostat (IEC Model Minotome Cryostat. International Equipment Co. 300 Second Avenue Needham Heights. Mass. 02194) and frozen to - 20°C. The 5 to 6 micron thick frozen tissue were cut at - 20°C and mounted on glass slide in a drop of phosphate buffer (pH 7.4). The wet slides were allowed to dry at room temperature for 1.0 minute and fixed in fixer (formal-ethanol 10 percent) for 2-3 minutes. The slides were gently rinsed with running tap water for 15 seconds and the wet slides were held at room temperature for 1.0 minute. These sections were dipped in freshly prepared incubation mixture (pH 5.8 to 6.5) for 1.0 minute at room temperature. The slides were washed in running tap water for 15 seconds and allowed to dry at room temperature. They were counter stained with hematoxylin and eosin stain and dried in the oven.

Four different fields per slide were examined under the microscope. The heterogenous myocyte injury, the number of leukocytes and extracellular granules were counted for each field. The number of observations were statistically analysed using student's t - test (Dospekhov, 1984); the mean, standard error and standard deviation per field for the infarcted and control dogs were determined.

Reagents for MPO Histochemical Staining:-

The reagents were prepared according to the procedure of (Kaplow, 1965).

Fixative: 10 percent formal-ethanol (10 ml of 37 percent formaldehyde and 90 ml of absolute ethyl alcohol).

The Incubation Mixture Was Prepared From the Following Reagents:-

1) 30 percent ethyl alcohol.
2) Benzidine dihydrochloride (Sigma Chemical Co. P.O. Box 14508 St. Louis. MO. 63178, U.S.A.).
3) 0.132M zinc sulphate (3.8 percent W/V Zn $SO_4.7H_2O$).
4) Sodium acetate ($NaC_2 H_2O_2 \cdot 3H_2O$).
5) 3 percent hydrogen peroxide.
6) 1.0N sodium hydroxide.

The Quantities of These Reagents Used Were As Follows:-

1) 30 percent ethyl alcohol: 33.3ml
2) Benzidine dihydrochloride: 0.1gm
3) 0.132M zinc sulphate (3.8 percent W/V) Zn $SO_4.7H_2O$: 0.33ml.
4) Sodium acetate ($NaC_2H_2O_2 \cdot 3H_2O$) : 0.33gm
5) 3 percent hydrogen peroxide: 0.23ml
6) 1.0N sodium hydroxide: 0.5ml

The final p^H was 6.0 $\pm$ 05. The reagents were added in the listed order, mixing well with each addition. The ability of the incubation mixture to demonstrate MPO was tested after its preparation by using fresh canine citrated peripheral blood smear. A fresh incubation mixture was prepared for every staining.

Staining Procedure:-

The staining procedure was carried out according to (Faplow, 1965) at room temperature.

1. Fresh citrated peripheral blood smear or frozen tissue section were used.
2. The blood smears were fixed for 1.0 minute and tissue sections for 2 to 3 minutes using 10 percent formal-ethanol. The slides were washed gently for 15 to 30 seconds with tap water. Excess water was shake off.
3. Wet slides were placed in a couplin jar containing incubation mixture for 1.0 minute.

4. The slides were washed gently for 15 seconds in running tap water, dried and examined under the microscope.

5. Frozen tissue sections were counter stained with hematoxylin and eosin stain.

Criteria for Evaluation of Myocytes Injury in heart Sections:-

Morphological studies: The criteria used by Rahamathulla and Ashraf (1984) were followed to evaluate the tissue sections.

The following score was used for classifying the degree of injury in the myocytes.

Injury Score

0 Compact fibers, presence of striations, uniform staining of nuclear material.

1 Weak striations, slight clumping and margination of chromatin, and presence of inter-fiber spaces. Minor injury.

2. Loss of structural details, homogenous cytoplasm , presence of few contracted myofibrils and remnants of nucleus. Moderate injury.

3 Marked change in structure, presence of contraction bands and loss of nuclei. Cell separation and rupture with loss of nuclei. Severe injury.

Method of Statistical Analysis:-

Student's t test.

According to Gomez and Gomez (1984), the t-test if the error degrees of freedom in the two groups differ, with ni d. f. for the i th group and nj d.f. for the j th group, the tabular t value is computed as: $t = \frac{S_i^2 t_i + S_j^2 t_j}{S_i^2 + S_j^2}$ where

ti and tj are the tabular values obtained from Distribution of t probability given by Fisher and Yates (1963) with ni

and nj degrees of freedom, respectively.

$$\text{Calculated } t = \frac{\bar{X}i - \bar{X}j}{SEi + SEj} \text{ where}$$

$\bar{X}i - \bar{X}j$ is the difference in mean between i and j groups divided by SEi + SEj, where SEi + SEj is the sum of the standard errors of i and j groups.

Method of Estimating Leukocytes Count in Heart Tissue:-

Leukocytes were estimated by direct count in four different fields per tissue section of the hearts under the microscope (X 40) and expressed as mean leukocytic count per microscopic field.

Method of Estimating Extracellular Granules in Heart Tissue:-

Extracellular granules were estimated by direct count in four different fields per tissue section of the hearts under the microscope (X 40) and expressed as mean extracellular granules count per microscopic field.

Identification of Granules:-

The granules are stained brown-yellow in fresh frozen section stained with MPO specific histochemical stain described by (Kaplow, 1965).

LIST OF EXPERIMENTAL ANIMALS

Animal Experiment 1

Reperfused dogs	Control dogs
1	9
3	11
4	17
7	2
8	
10	
12	
13	
14	
15	
16	

Animal Experiment 2

Reperfused dogs	Control dogs
1	1C
2	2C
3	
6	
7	
8	
9	
11	
14	
15	
16	
17	
19	
20	
21	
22	

RESULTS

Gross Appearance of the Heart After Ligation of Left Descending Branch of Circumflex Coronary Artery (LAD).

On ligation of LAD, the lateral and posterior surfaces of the left ventricle at the areas at risk became markedly cyanotic extending from the point of ligation to the apex of the left ventricle. There were scattered petecheal hemorrhages on the surface of the areas at risk. The heart on the whole appeared moderately dilated especially at the time when the ligature was about to be removed, i.e. at the end of 1.0 hour. The 9 dogs which died during surgery showed severe ventricular fibrillation before death.

Gross Appearance of Reperfused Hearts at End of Study.

The hearts of the dogs maintained for 1 day and 7 days after surgery revealed the same gross appearance. There were large light-tan reperfused zones in the areas that appeared cyanotic during surgery. The reperfused hearts were severely dilated, flabby and rounded (Figure 1). The walls of the ventricles were thickened. The reperfused area involved the entire thickeness of the left ventricle, myocardium including the posterior papillary muscle. No such observations were noticed in the hearts of the control animals where LAD was not ligated (Figure 2).

Microscopic Appearance:-

Microscopic examination of the H & E sections from the reperfused area of the left ventricle from a dog maintained for 7 days after surgery showed heterogenous degenerative changes in the subepicardial zone and uniform necrosis in the subendocardial zone. In all the cases of LAD ligation, the posterior papillary muscle was entirely necrotic except for a very thin layer of non



Figure 1. Gross picture of the heart of a dog maintained for 1 day after surgery. Showing a dilated, flabby and rounded appearance.



Figure 2. Gross picture of the heart of a control dog with shams operation maintained for 1 day after surgery. No gross pathological changes are seen.

necrotic myocardium beneath the endocardium. Leukocytic infiltration consisting of neutrophils, lymphocytes, plasma cells was observed (Figure 3). There was haemorrhage in the necrotized areas (Figure 4). Evidence of similar histopathological change was present in the left ventricle of the hearts outside the risk area.

Similar histopathological lesions were observed in the reperfused area of hearts of dogs maintained for 1 day.

In sections of hearts reperfused for 1 day, there was edema of some myocytes which were wider than normal. There were necrotic myocardial fibres. The infiltrated inflammatory cells were mainly neutrophils with few lymphocytes.

In sections of hearts reperfused for 7 days, there were more necrotic myocardial fibres with lost of nuclei and striations. The infiltrated inflammatory cells were neutrophils, and more lymphocytes than in sections of hearts reperfused for 1 day. In addition, there were monocytes and few plasma cells which were not seen in sections of hearts reperfused for 1 day. However, the control dogs of both groups did not show any significant histopathological change (Figure 5).

Extracellular Granules:-

These are lysosomal granules released from the stimulated neutrophils and monocytes into the extracellular space. MPO was detected on histochemical staining of the granules. The myocytes close to these granules revealed various degrees of cell injury in the reperfused area in dogs maintained 1 and 7 days post surgery (Figure 6).

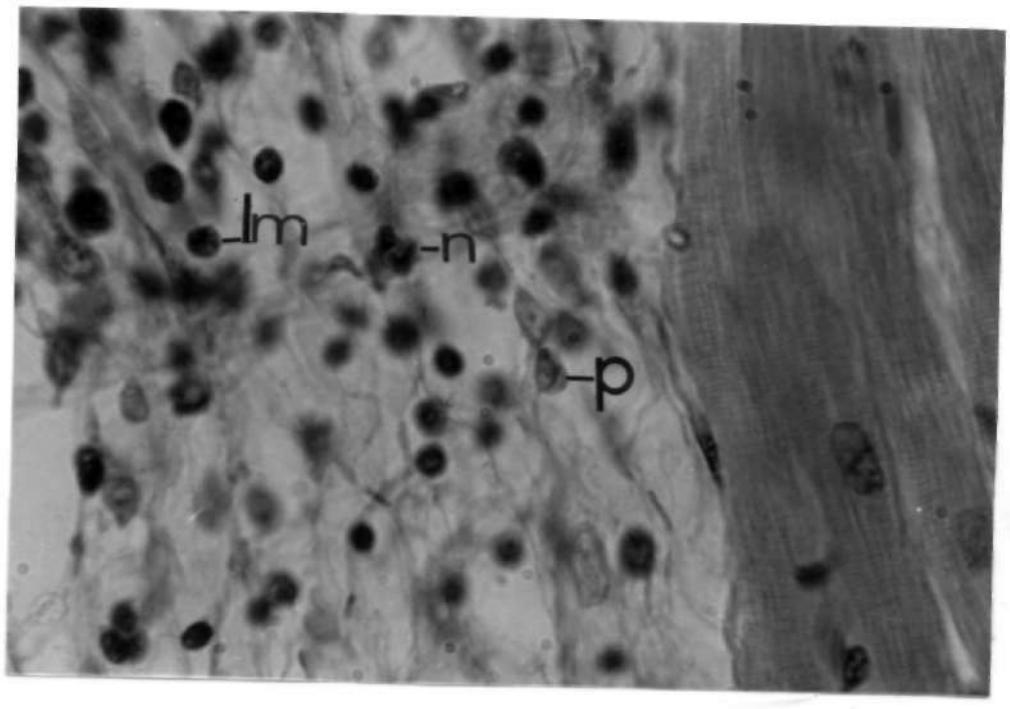


Figure 3. Photomicrograph of a section of the heart showing neutrophil (n), lymphocyte (lm) and plasma cell (p). H and E X 100.

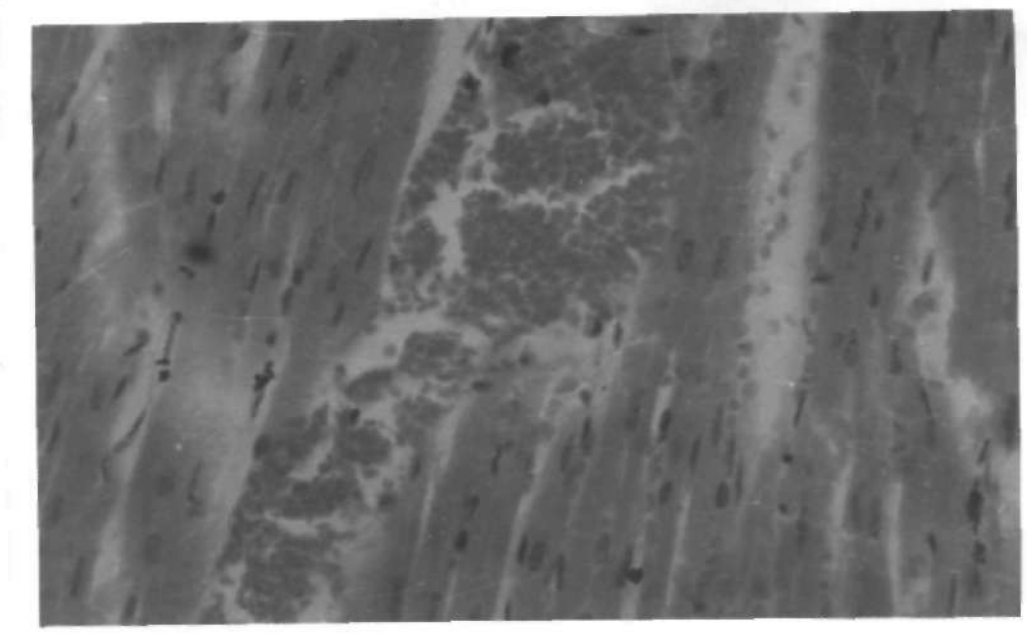


Figure 4. Photomicrograph of the section of the heart showing haemorrhage in the necrotized area.
H and E X 40.

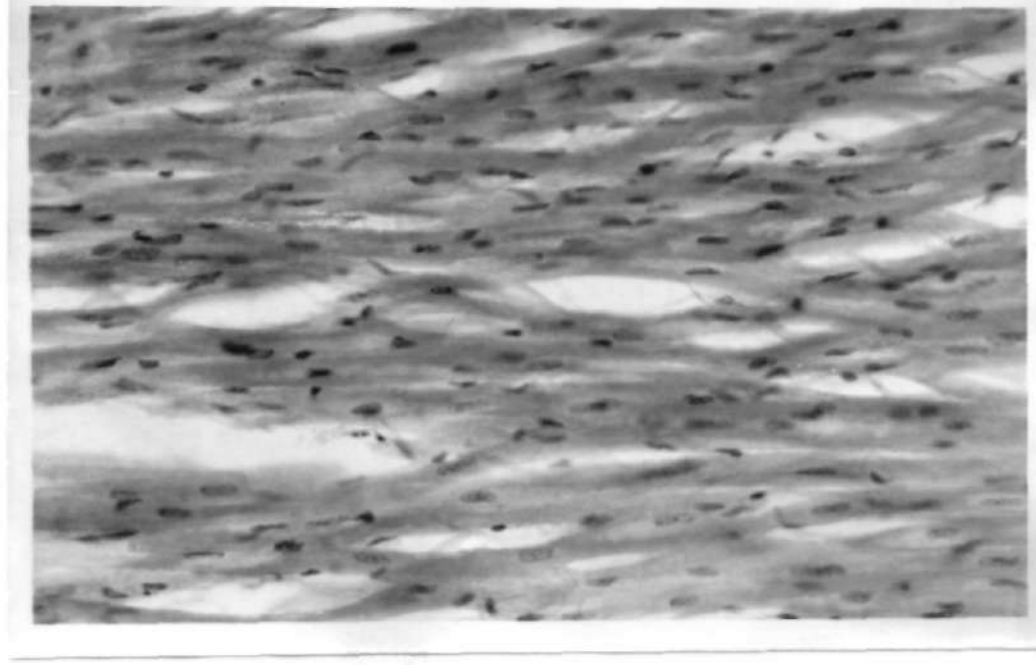


Figure 5. Photomicrograph of the section of the heart of a control dog with shams operation maintained for 7 days after surgery. No significant histopathological change. H and E X 40.

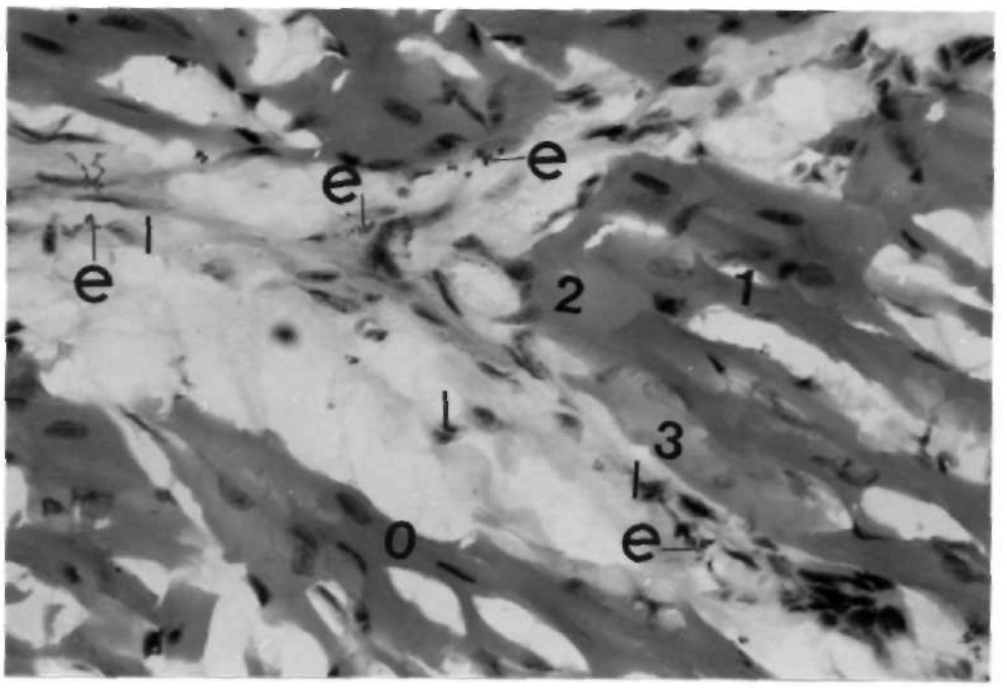


Figure 6. Photomicrograph of frozen section of the heart from a dog maintained for 7 days after surgery showing leukocytes (l) extracellular granules (e) myocytes with score 0, 1, 2 and 3. Histochemical stain X 40.

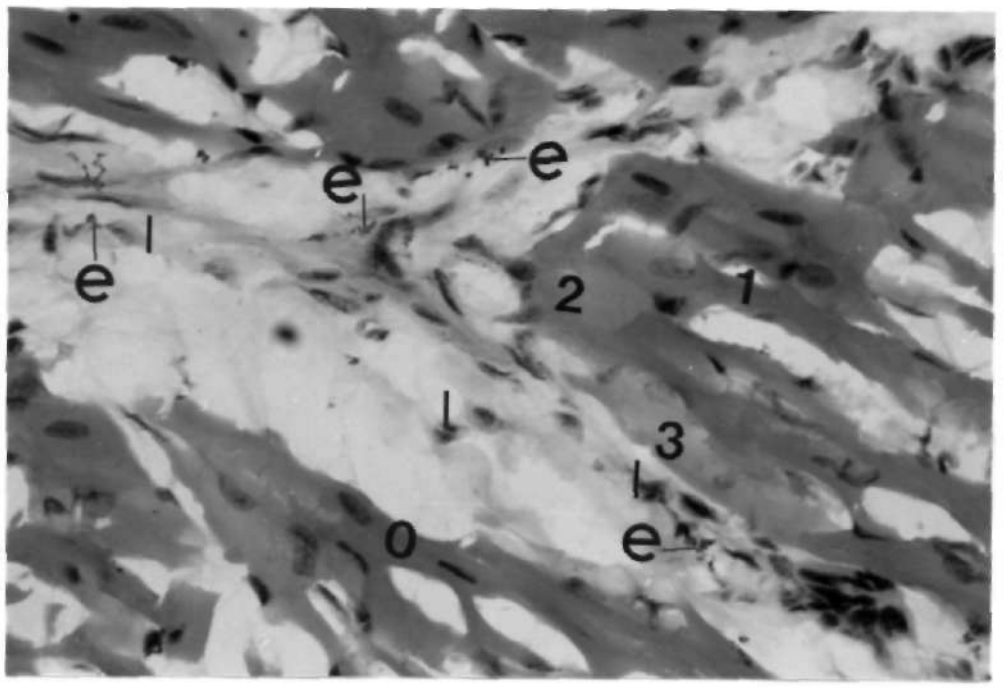


Figure 6. Photomicrograph of frozen section of the heart from a dog maintained for 7 days after surgery showing leukocytes (1) extracellular granules (e) myocytes with score 0, 1, 2 and 3. Histochemical stain X 40.

In the peripheral blood smears of non-operated as well as operated dogs, MPO was observed in the intracellular granules of neutrophils on histochemical staining (Figure 7), while on staining with Giemsa no such reaction was seen (Figure 8). Eosinophils also reacted for MPO whereas lymphocytes and platelets did not react on histochemical staining.

The myocytes of sections of hearts reperfused for 1 day revealed heterogenous injury. As summarised in Table 1A, those myocytes with an injury score of 1 predominated, followed by those with injury score of 2 (Figure 9). In the control dogs, only a negligible number of myocytes had an injury score of 1. Compared to control dogs, the injury score recorded for myocytes from reperfused area of the heart were statistically significant ($P<0.05$) in case of 0 score, and ($P<0.01$) in case of injury scores 1, 2 and 3.

The myocytes of sections of hearts reperfused for 7 days revealed heterogenous injury. As summarised in Table 1B, those myocytes with an injury score of 2 predominated, followed by those with injury score of 3 (Figure 10). In the control dogs, there were no myocytes with injury i.e. score 0. Compared to control dogs, the injury scores recorded for myocytes from reperfused area of the heart were statistically highly significant ($P<0.01$) in scores 0, 1, 2 and 3.

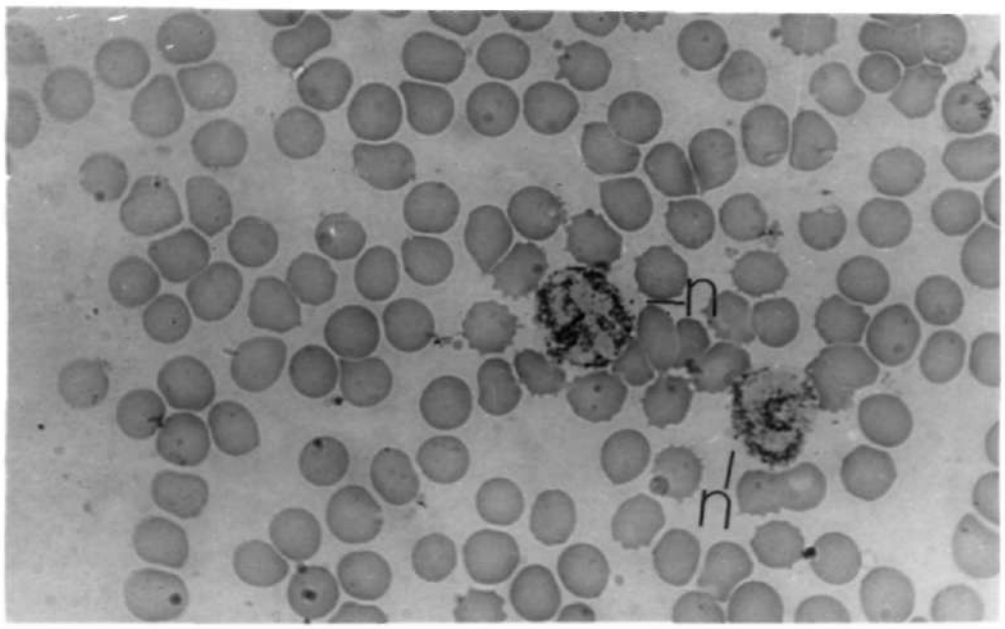


Figure 7. Photomicrograph of the canine peripheral blood smear showing MPO in intracellular granules (i) of the neutrophils (n). Histochemical stain X 100.

TABLE 1

Table 1A: Mean Myocyte Injury Score in the Heart Sections from Dogs Maintained 1 Day Post Surgery.

Injury score	Reperfused dogs(11)		Control dogs (4)	
	Mean	SE	Mean	SE
0	1.2*	0.62	65 .71	3.01
1	47.82**	5.96	0.87	0.86
2	27.11**	3.03	0	0
3	0.45**	0.45	0	0

SE = Standard Error, * = P≤0.05, ** = P≤0.01

Table 1B: Mean Myocyte Injury Score in the Heart Sections from Dogs Maintained 7 Days Post Surgery.

Injury score	Reperfused dogs(16)		Control dogs (2)	
	Mean	SE	Mean	SE
0	11.34**	2.17	116.53	3.79
1	13.53**	1.86	0	0
2	51.14**	2.63	0	0
3	15.3**	2.15	0	0

SE = Standard Error, ** = P≤0.01

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Table 1B: Mean Myocyte Injury Score in the Heart Sections from Dogs Maintained 7 Days Post Surgery.

Injury score	Reperfused dogs(16)		Control dogs (2)	
	Mean	SE	Mean	SE
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1	13.53**	1.86	0	0
2	51.14**	2.63	0	0
3	15.3**	2.15	0	0

SE = Standard Error, ** = P<0.01

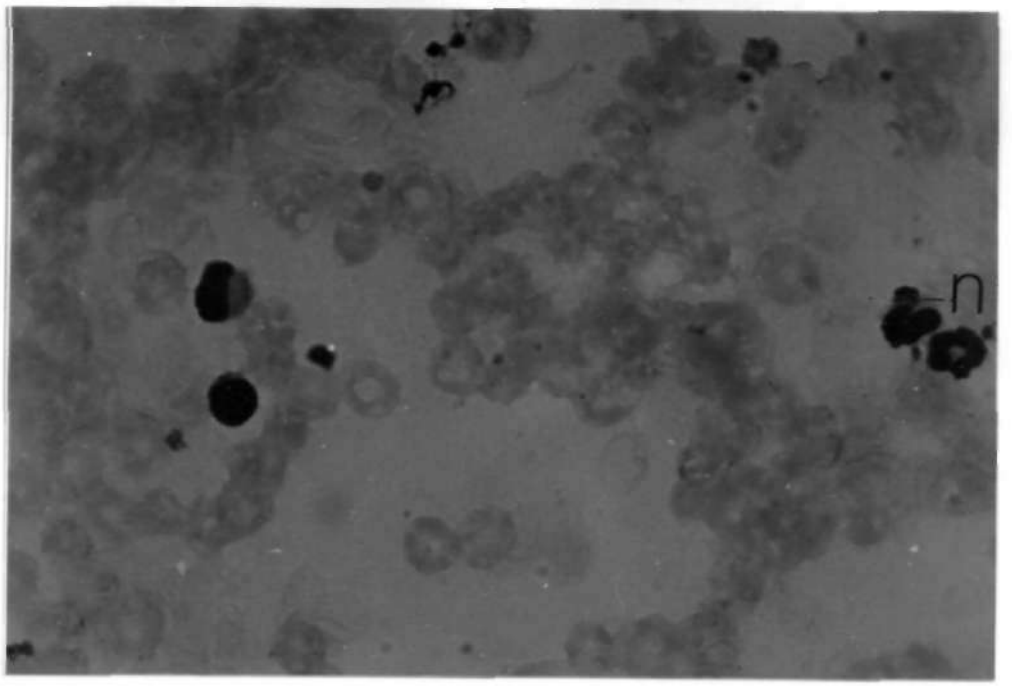


Figure 8. Photomicrograph of the canine peripheral blood smear showing no MPO in the neutrophil (n) as observed in Figure 7. Giemsa stain X 100.

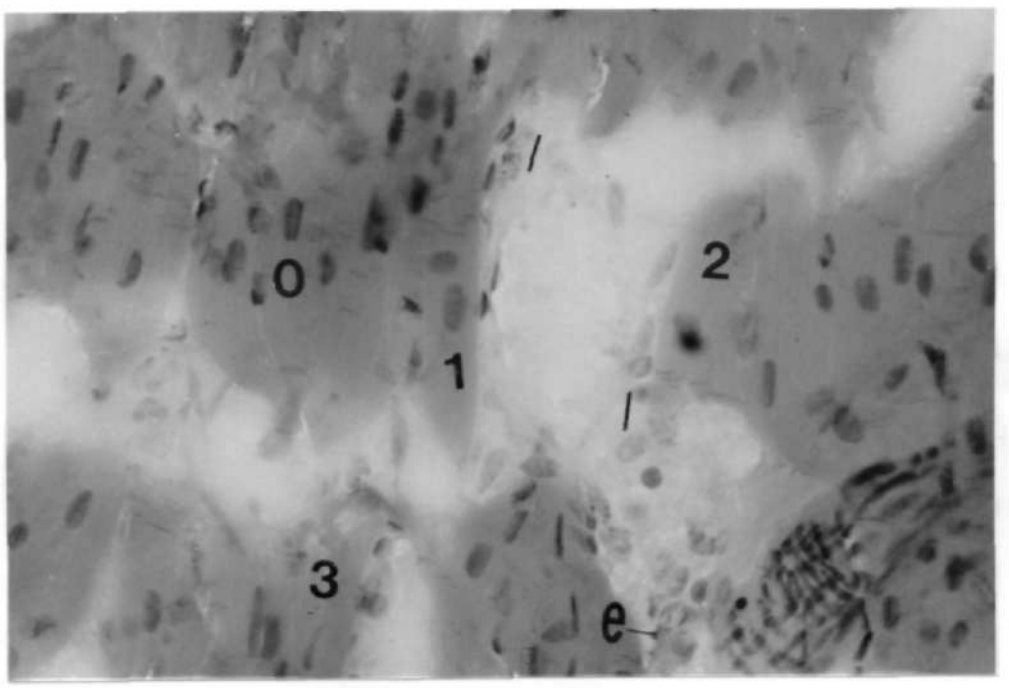


Figure 9. Photomicrograph of frozen section of a heart from a dog maintained 1 day after surgery showing myocytes of scores 0, 1, 2 and 3; leukocytes (l) extracellular granule (e) Histochemical stain X 40.

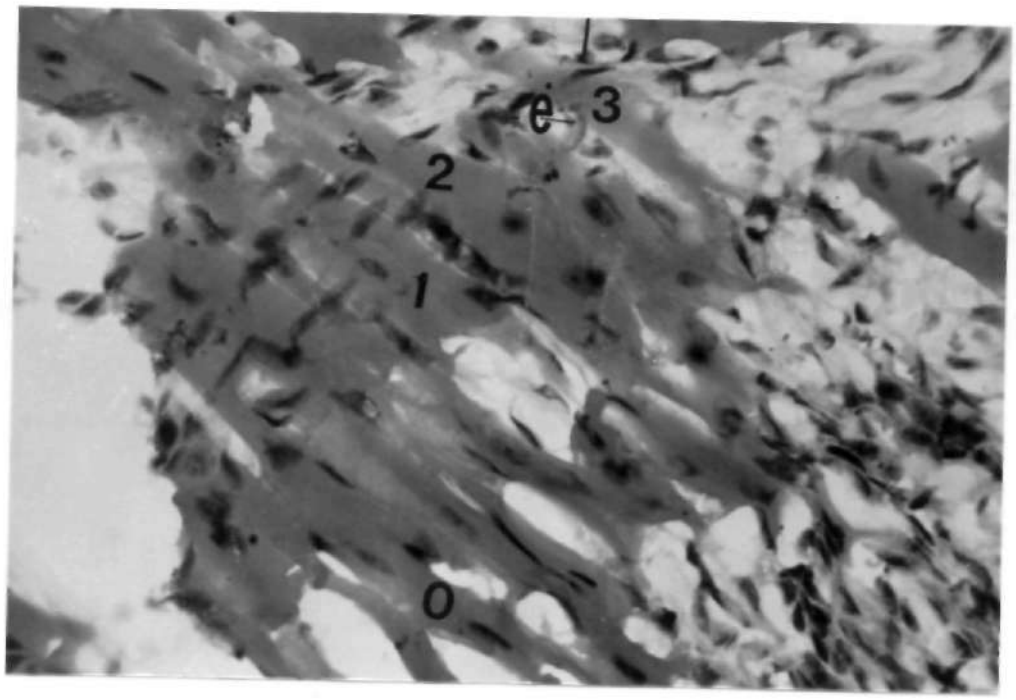


Figure 10. Photomicrograph of frozen section of the heart from a dog maintained for 7 days after surgery showing myocytes of scores 0, 1, 2 and 3; leukocytes (1) extracellular granules (e).
Histochemical stain X 40.

As summarised in Table 2A, the leukocytes count in sections of hearts reperfused for 1 day was highest in areas where there were myocytes with injury score 2, followed by areas where there were myocytes with injury score 1 (Figure 11). In the control dogs leukocytes were rarely seen. Compared to control dogs, the leukocytes count from reperfused area of the heart were statistically significant ($P<0.05$) in case of 0 score, and ($P<0.01$) in case of injury scores 1, 2 and 3.

As summarised in Table 2B, the leukocytes count in sections of hearts reperfused for 7 days was highest in areas where there were myocytes with injury score 3, followed by areas where there were myocytes with injury score 2.(Figure 12). In the control dogs, very few leukocytes were seen. Compared to control dogs the leukocytes count from reperfused area of the heart were statistically not significant ($P>0.05$) in case of score 0, and ($P<0.01$) in case of injury scores 1, 2 and 3.

TABLE 2

Table 2A: Mean Leukocytic Count per Microscopic Field with Relationship to Myocyte Injury in the Heart Sections from Dogs Maintained 1 Day Post Surgery.

Injury score	Reperfused dogs (11)		Control dogs (4)	
	Mean	SE	Mean	SE
0	0.02*	0.02	1.17	0.18
1	3.76**	0.63	0	0
2	6.82**	0.84	0	0
3	0.25**	0.26	0	0

SE = Standard Error, * = $P<0.05$, ** = $P<0.01$

Table 2B: Mean Leukocytic Count per Microscopic Field with Relationship to Myocyte Injury in the Heart Sections from Dogs Maintained 7 Days Post Surgery.

Injury score	Reperfused dogs (16)		Control dogs (2)	
	Mean	SE	Mean	SE
0	0.07NS	0.03	0.12	0.05
1	1.04**	0.16	0	0
2	9.94**	0.81	0	0
3	22.54**	1.00	0	0

SE = Standard Error, NS = Not significant at $P>0.05$
** = $P<0.01$

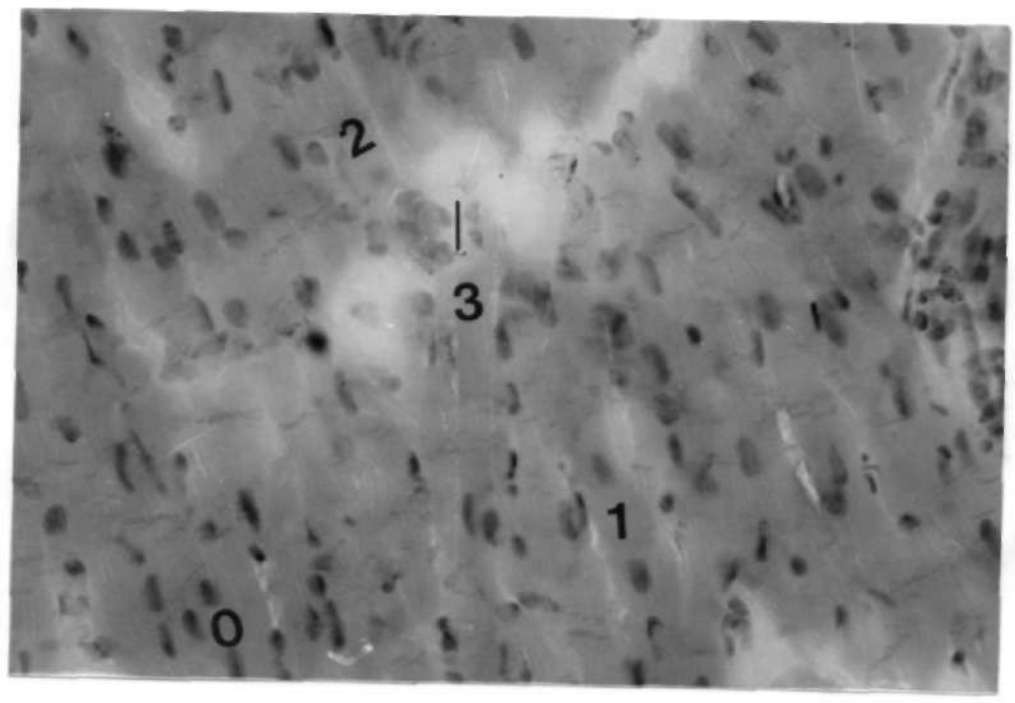


Figure 11. Photomicrograph of frozen section of the heart of a dog maintained for 1 day after surgery showing myocytes with scores 0, 1, 2 and 3; leukocytes (1). Histochemical stain X 40.

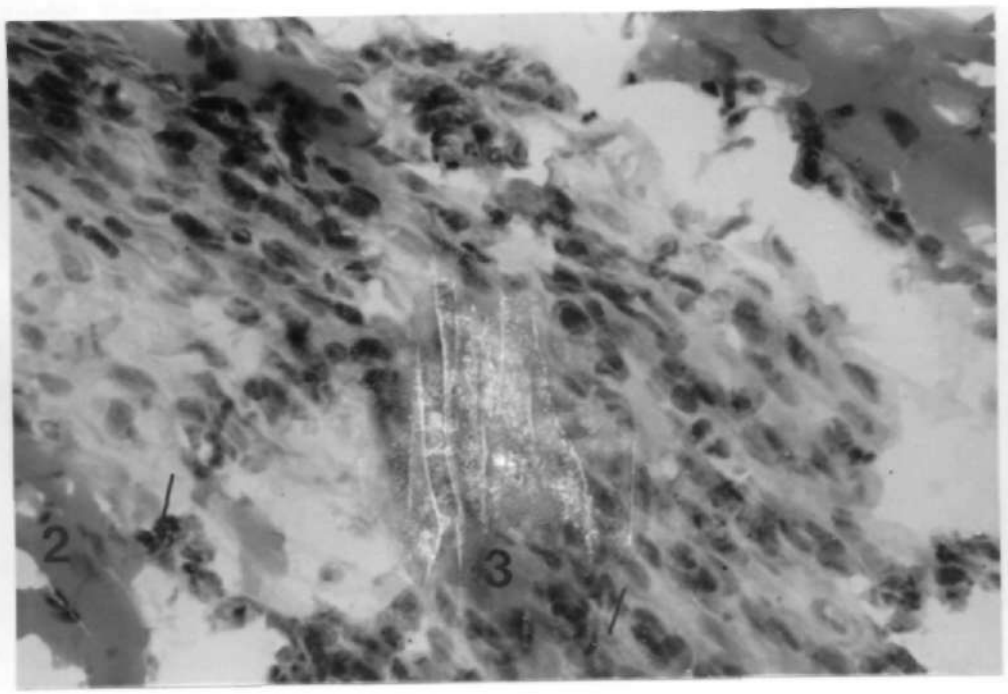


Figure 12. Photomicrograph of frozen section of the heart of a dog maintained for 7 days after surgery showing myocytes with score 2, 3; aggregated leukocytes (1). Histochemical stain X 40.

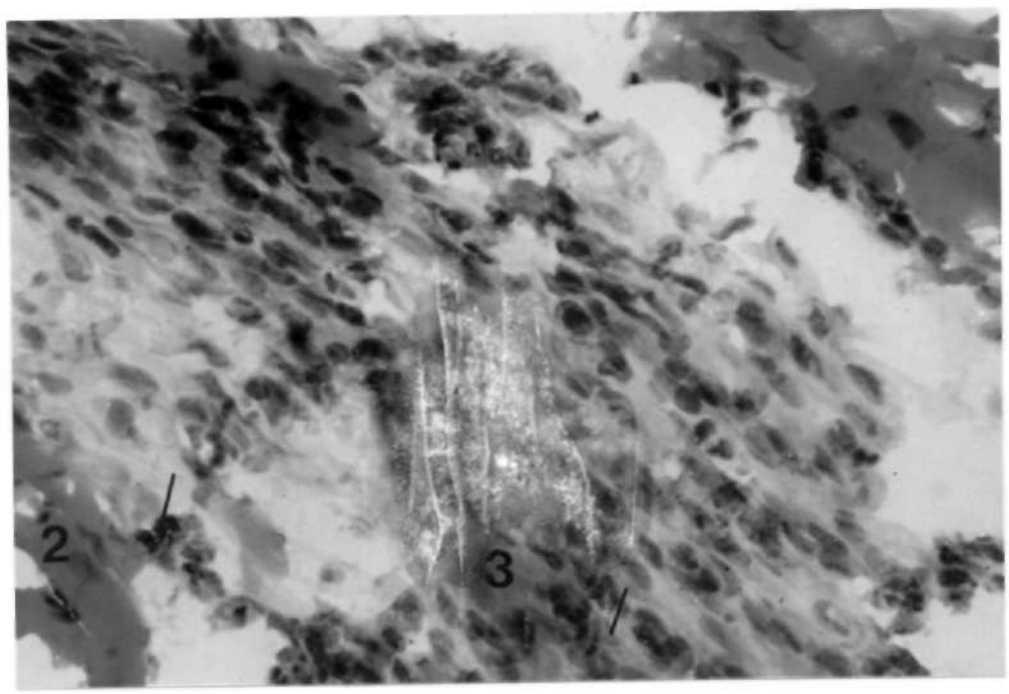


Figure 12. Photomicrograph of frozen section of the heart of a dog maintained for 7 days after surgery showing myocytes with score 2, 3; aggregated leukocytes (1). Histochemical stain X 40.

The extracellular granules in sections of hearts reperfused for 1 day were more in areas where there were myocytes with injury score 2, followed by areas where there were myocytes with injury score 1 as summarised in Table 3A (Figure 13). In the control dogs, there were very negligible extracellular granules in areas with score 1, and there were no extracellular granules in areas with 0 score. Compared to control dogs, the extracellular granules count from reperfused area of the heart were statistically not significant ($P > 0.05$) in case of scores 0 and 1, and ($P < 0.01$) in case of injury scores 2 and 3.

As summarised in Table 3B, the extracellular granules in sections of hearts reperfused for 7 days were mostly seen in areas where there were myocytes with injury score 3, followed by areas where there were myocytes with injury score 2 (Figure 6). In the control dogs, there were no extracellular granules. Compared to control dogs, the extracellular granules count from reperfused area of the heart were statistically not significant ($P > 0.05$) in case of score 0, and ($P < 0.01$) in case of injury scores 1, 2 and 3.

TABLE 3

Table 3A: Mean Extracellular Granules Count per Microscopic Field with Relationship to Myocyte Injury in the Heart Sections from Dogs Maintained 1 Day Post Surgery.

Injury score	Reperfused dogs (11)		Control dogs (4)	
	Mean	SE	Mean	SE
0	0 NS	0	0	0
1	0.12 NS	0.04	0.06	0.06
2	0.61 **	0.14	0	0
3	0.02 **	0.02	0	0

SE = Standard Error, NS = Not significant at $P > 0.05$,
** = $P < 0.01$

Table 3B: Mean Extracellular Granules Count per Microscopic Field with Relationship to Myocyte Injury in the Heart Sections from Dogs Maintained 7 Days Post Surgery

Injury score	Reperfused dogs(16)		Control dogs(2)	
	Mean	SE	Mean	SE
0	0 NS	0	0	0
1	0.11 **	0.03	0	0
2	1.17 **	0.097	0	0
3	1.88 **	0.27	0	0

SE = Standard Error, NS = Not significant at $P > 0.05$,
** = $P < 0.01$

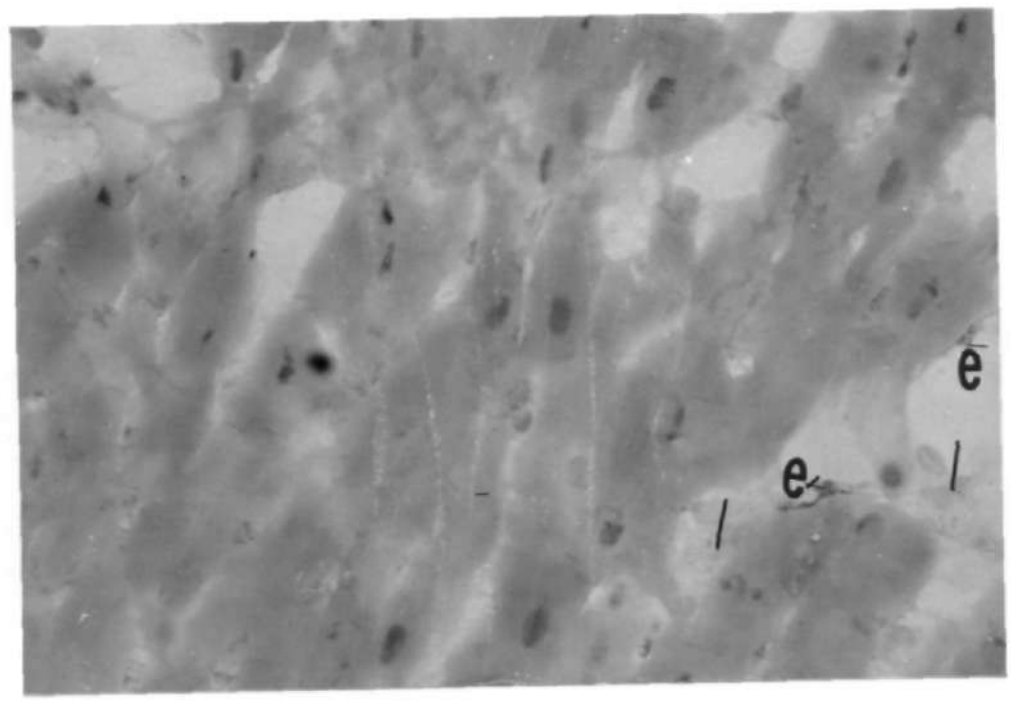


Figure 13. Photomicrograph of frozen section of the heart from a dog maintained for 1 day after surgery showing leukocytes (l) extracellular granules (e). Histochemical stain X 40.

In sections of hearts reperfused for 1 day, the myocytes with injury score 1 were most prevelent, followed by myocytes with injury score 2. In sections of hearts reperfused for 7 days, myocytes with injury score 2 predominated. followed by myocytes with injury score 3 as summarised in Table C. In sections of hearts reperfused for 1 day compared to sections of hearts reperfused for 7 days, the recorded myocytes injury scores were statistically not significant ($P>0.05$) in case of 0 score, and ($P<0.01$) in case of injury scores 1, 2 and 3.

The leukocytes in sections of hearts reperfused for 1 day were mostly seen in areas where there were myocytes with injury score 1, followed by areas where there were myocytes with injury score 2. In sections of hearts reperfused for 7 days, leukocytes were predominantly seen in areas where there were myocytes with injury score 3, followed by areas where there were myocytes with injury score 2 as summarised in Table D. In sections of hearts reperfused for 1 day compared to sections of hearts reperfused for 7 days, leukocytes count was statistically not significant ($P>0.05$) in case of 0 score, and ($P<0.01$) in case of injury score 1, 2 and 3.

TABLE C : Mean Myocytes Injury per Microscopic Field (X 40)

Relative score of myocytes injury	1 Day reperfused hearts(11)		7 Days reperfused hearts(16)	
	Mean	SE	Mean	SE
0	3.453^{NS}	1.198 (n=442)	1.368	0.346 (n=383)
1	42.117**	2.785 (n=5391)	3.436	0.749 (n=960)
2	12.133**	2.125 (n=1553)	39.689	1.649 (n=11113)
3	0.961**	0.241 (n=123)	5.739	0.501 (n=1607)

SE = Standard Error, n= number of myocytes counted,

NS = Not significant at P$>$0.05, ** = P$<$0.01.

TABLE D: Mean Leukocytic Count per Microscopic Field (X 40) in Relation to Myocytes Injury.

Relative score of myocyte injury	1 Day reperfused hearts (11)		7 Days reperfused hearts(16)	
	Mean	SE	Mean	SE
0	0^{NS}	0 (n=o)	0	0 (n=o)
1	7.039**	0.549 (n=901)	0.911	0.233 (n=255)
2	5.938**	0.999 (n=760)	21.982	1.022 (n=6155)
3	3.695**	0.905 (n=473)	27.736	3.117 (n=7766)

SE = Standard Error, n = number of leukocytes counted,

NS = Not significant at P$>$0.05, ** = P$<$0.01.

In sections of hearts reperfused for 1 day, extracellular granules were mostly seen in areas where there were myocytes with injury score 3, followed by areas where there were myocytes with injury score 2. In sections of hearts reperfused for 7 days, extracellular granules were predominantly seen in areas where there were myocytes with injury score 3, followed by areas where there were myocytes with injury score 2 as summarised in Table E. In sections of hearts reperfused for 1 day compared to sections of hearts reperfused for 7 days, the extracellular granules count was statistically not significant ($P > 0.05$) in case of scores 0, 1 and 2, and ($P < 0.01$) in case of injury score 3.

TABLE E

Mean Extracellular Granules Count per Microscopic Field (X 40)

in Relation to Myocytes Injury.

Relative score of myocyte injury	1 Day reperfused hearts (11)		7 Days reperfused hearts (16)	
	Mean	SE	Mean	SE
0	0^{NS}	0. (n=0)	0	0 (n=0)
1	0.086^{NS}	0.067 (n=11)	0.096	0.036 (n=27)
2	0.961^{NS}	0.527 (n=123)	2.35	0.248 (n=658)
3	1.32^{**}	0.473 (n=169)	3.993	0.535(n=1118)

SE = Standard Error, n = number of extracellular granules counted,

NS = Not significant at $P > 0.05$, ** = $P < 0.01$.

DISCUSSION

The results of this study showed that in ischemia/reperfusion injury the myocytes of sections of dog heart undergo heterogenous injury that was classified into 4 identifiable types depending on cellular alterations. They varied from normal to extremely damaged myocytes. This finding confirms previous reports by Jennings et al. (1957), Rahamathulla and Ashraf (1984), and Rahamathulla et al. (1985).

According to several investigators, the infiltration of neutrophils into ischaemic myocardium exacerbates myocardial damage upon reperfusion, Romson et al., 1982, 1983, 1984; Bednar et al., 1985; Hess et al., 1985; Mullane et al., 1985; Engler et al., 1986), while drugs that inhibits neutrophil activity or function reduce infarct size. In this study, there was increased severity of myocyte injury with corresponding aggregation of leukocytes in reperfused areas.

Demonstration of MPO using MPO specific histochemical stain confirmed the presence and actual involvement of hydrogen peroxide in causing reperfusion injury and myocardial dysfunction.

Mullane et al. (1985) and Bedner et al. (1985) assayed myeloperoxidase activity in reperfused hearts of dogs and emphasized that the activity of myeloperoxidase can be used as an index of neutrophil infiltration into post-ischaemic reperfused heart. This study also confirmed that the activity of myeloperoxidase can serve as an index of infiltration by neutrophil into post ischaemic reperfused heart (Tables G and H). This was confirmed by histochemically localizing MPO in the post-ischaemic reperfused heart using MPO specific histochemical stain.

The results of this study showed that post-ischemic reperfusion injury is a dynamic process that requires close relationship between the myocytes, aggregation of leukocytes and extracellular granules released by the infiltrated leukocytes. Such a relationship is required for reperfusion injury to occur. This close relationship was not highlighted in the review of ischemia/reperfusion injury published by (Hess and Manson, 1984) and in the investigation conducted by Myron (1987) on reperfusion and reperfusion injury.

Myron (1987) reported that following coronary artery occlusion the extent of myocardial necrosis is determined by both duration of ischemia and the severity of ischemia. However, with limited duration and severity of ischemia, substantial myocardial injury may occur upon reperfusion. This study has shown that there was less myocardial necrosis in sections of hearts reperfused for 1 day, and there was more myocardial necrosis in sections of hearts reperfused for 7 days. The edema and cellswelling observed in the myocytes in this study was reported by other investigators (Englar et al., 1986; Myron, 1987).

Neutrophils accumulation during acute inflammatory response was reported earlier (Romson et al., 1983; Hess et al., 1985) in canine myocardial ischemia/reperfusion injury. In this study, neutrophils were the main inflammatory cells present in sections of hearts reperfused for 1 day. In sections of hearts reperfused for 7 days neutrophils were the most predominant inflammatory cells followed by lymphocytes, monocytes and plasma cells. This result

is supported by reported findings implicating role of leukocytes in acute and or chronic inflammatory responses (Ward et al., 1979; Ward, 1980). It is also supported by the reported findings of Hess et al., (1985) that polymorphonuclear leukocytes migrate into the ischemic area within hours of the initial insult, initiating acute inflammatory response to tissue necrosis.

Stimulated neutrophils transform molecular oxygen into highly reactive and cytotoxic free radicals superoxide, hydroxyl radical and hydrogen peroxide. When not limited to phagocytic vacuoles, these oxygen radicals degrade extracellular macromolecules, attack membrane phospholipids and promote cell injury or death (Del Maestro et al., 1980). In addition, release of the enzymatic contents of neutrophil granules into extracellular space may result in proteolytic attack on viable tissue (Weissman et al., 1980). This study, revealed that in sections of hearts reperfused for 1 day and sections of hearts reperfused for 7 days there were more infiltrated leukocytes and extracellular granules with corresponding increase in severity of myocytes injury from minor to severe injury in sections of hearts reperfused for 7 days, than in sections of hearts reperfused for 1 day (Tables C, D, E and F). These findings are supported by the reported cases above (Del Maestro et al., 1980; Weissman et al. 1980).

The findings from the present study are supported by reported findings of the investigations by Weiss and Lobuglio (1982) and Romson et al., (1983) that during the early acute

TABLE F

Relationship of Number of Leukocytes and Extracellular Granules in 1 Day and 7 Days Reperfused Heart Sections to the Degree of Myocyte Injury Scores.

Relative Score of myocyte injury	1 Day reperfused hearts (11)		7 Days reperfused hearts (16)	
	Number of leukocytes	Number of extracellular granules	Number of leukocytes	Number of extracellular granules
0	0	0	0	0
1	901	11	255	27
2	760	123	6155	658
3	473	169	7766	1118

inflammatory response, polymorphonuclear neutrophils undergo a complex series of functional and biochemical alterations that promote tissue lysis. It includes generation of cytotoxic hypochlorous acid via MPO - H_2O_2 - halide system. Although these events are important to the repair process, they may also result in the destruction of potentially viable tissue elements (Romson et al., 1983).

REFERENCES

Agner, K. (1941). Verdoperoxidase: a ferment isolated from leukocytes. Acta Physiologica Scandinavica, 2 (suppl. 8): 1.

Agner, K. (1958). Crystalline myeloperoxidase. Acta Chemica Scandinavica, 12: 89

Altschuld, R.A., Hosteltler, J.R. and Brierly, G.P. (1981). Response of isolated rat heart cells to hypoxia, reoxygenation and acidosis. Circulation Research, 49: 307-316.

Ashraf, M. and Rahamathulla, P.M. (1984). Cardiac injury in short duration anoxia and modification by diltiazem, a calcium chennel blocking agent. Journal of American Collage Cardiology, 3: 1237-1244.

Autor, A.P. (1974). Reducation of paraquat toxicity by superoxide dismutase. Life Sciences, 14: 1309-1310.

Badwey, J.A. and Karnowsky, M.L. (1980). Active oxygen species and the functions of phagocytic leukocytes. Annal Reverend Biochem, 49: 695-726.

Balazes, E.A., Davis, J.V., Phillips, G.O. and Young, M.D. (1967). Transient intermediates in the radiolysis of hyaluronic acid. Radiation Research, 31: 243-255.

Beauchamp, C. and Fridovich, I. (1970). A mechanism for the production of ethylene from methional. Journal of Biological Chemistry, 245: 4641.

Bednar, M., Smith, B., Pinto, A. and Mullane, K.M. (1985). Nafazatrom - induced salvage of ischemic myocardium in anaesthetized dogs is mediated through inhibition of neutrophil function. Circulation Research, 57: 131-140.

Bouchary, B. and Manjo, G. (1974). Histopathology of early myocardial infarctm. A new approach. American Journal of Pathology, 74: 301-312.

Boveris, A. (1977). Mitochondrial production of superoxide radical and hydrogen peroxide. In: Oxygen and Physiological functions. (ed) J.O. Bsis, F.F. Professional information library. Pryor, W.A. (1966) Free Radicals. McGraw-Hill, New York, pp.67-82.

Charles, C.T. (1968). Pathology of the heart and blood vessels. 3rd Ed. (ed). Gould, S.E., Charles, C. Thomas. Publishers Springfield, Illinois, United State of America, pp. 606-611.

Critchley, M. (1978). Butter worth medical dictionary. 2nd Ed. (ed) Macdonald Critchley, Butter-worths, London, Boston, pp. 884.

Del Maestro, R.F. (1979). The influence of oxygen derived free radicals on in vitro and in vivo model systems. Interactions of superoxide anion radical, hydrogen peroxide and hydroxly radical. Diss. Acta Universitatis Upsaliensis., Uppsala, Abstract. 340.

Del Maestro, R.F., Arfors, K.E. and Lindblom, R.R. (1979). Free radical depolymerixation of hyaluronic acid. Influence of scavenger substances. In: 10th European Conference. Microcirculation, Cagliari 1978. Bibl anat (ed.) Lewis, D.H., Kargar, Basel. No.18 pp 132-135.

Del Maestro, R.F. (1980). An approach to free radicals in medicine and biology. Acta physiologica scandinavica Supply, 492; 153-168.

Detweiler, D.K., Patterson, D.F., Luginbuhl, H., Rhodes, W.H., Buchanan, J.W., Knight, D.H., and Hill, J.D. (1968). Diseases of cardiovascular system. In Canine medicine. Ed. E.J. Catcott. 1st Catcott (ed). American Veterinary Publications, Inc., Santa Barbara, California, pp. 589.

Dospekhov, B.A. (1984). Field experimentation statistical procedures. Mir Publichers pp 175-178.

Dunn, W.B., Haedin, J.H. and Spicer, S.S. (1968). Ultrastructural localization of myeloperoxidase in human neutrophil and rabbit heterophil and eosinophil leukocytes. Blood, 32: 935-944.

Ehrenberg, A. and Agner, K. (1958). The molecular weight of myeloperoxidase. Acta Chemica Scandinavica, 12: 95-100.

Ellison, G.W. (1980). Vascular ring anomalies in the dog and cat. Continuing Education Article 2 Vol.11, No.9. pp 693.

Engler, R.L., Dahlgren, M.D., Morris, D.D. et al. (1986). Role of leukocytes in response to acute myocardial ischemia and reflow in dog. American Journal of physiology, 251 (Heart Circulation Physiology 20): H314-H322.

Fisher, R.A. and Yates, F. (1963). Statistical tables for biological, agricultural, and medical research. 6th Ed. Oliver and Boyd, pp 156.

Fleming, J.S. and Braimbridge, M.V. (1974). Lecture notes on cardiology. 3rd. Ed. Blackwell Scientific Publications, Oxford, Edinburgh Melbourne, pp 276-282.

Fong, K.L., McCay, P.B., Royer, J.L. Keele, B.B. and Misra, H. (1973). Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. Journal of Biological Chemistry, 248: 7792-7797.

Fridovich, I. (1976). Oxygen radicals, hydrogen peroxide and oxygen toxicity. In: Free radicals in biology, Volume 1. (ed) Pryor, W.A., Academic Press, New York, pp 239.

Fridovich, I. (1978). The biology of oxygen radicals. Science, 201: 875-880.

Gould, S.E. (1968). Pathology of the heart and blood vesels. 3rd Ed. Spring-field, 111, Charles C. Thomas Publishers, U. S. A. pp 601-648.

Goldstein, I.M. and Weissman, G. (1977). Effects of the generation of superoxide anion on permeability of liposomes. Biochemical and Biophysical Research Communication, 75: 604-609.

Gomez, K.A. and Gomez, A.A. (1984). Statistical procedures for agricultural research. 2nd. Ed. Wiley-Interscience Publication, John Willey and Sons, New York, pp 314-315.

Greenwald, R.A. and Moy, W.W. (1979). Inhibition of collagen gelation by action of the superoxide radical. Arthritis Rheum, 22: 251-259.

Haglund, U. and Lundgren, O. (1978). Intestinal ischemia and shock factors. Federation proceedings, 37: 2729-2733.

Halliwel, B. (1978). Biochemical mechanism accounting for the toxic action of oxygen on living organisms. The key role of superoxide dismutase. Cell Biology Int. Rep., 2: 113-128.

Harrison, J.E. and Schultz, J. (1976). Studies on chlorinating activity of myeloperoxidase. Journal of Biological Chemistry, 251: 1371.

Hearse, D.J., Humphrey, S.M. and Chain, E.B. (1973). Abrupt reoxygenation of the anoxic potassium-arrested reperfused rat heart: A study of myocardial enzyme release. Journal of Molecular Cell Cardiology, 5: 395-407.

Hearse, D.J. and Humphrey, S.M. (1975). Enzyme release during myocardial anoxia: a study of metabolic protection. Journal of Molecular cell Cardiology, 7: 463-482.

Hearse, D.J., Humphrey, S.M. and Bullock, G.R. (1978). The oxygen paradox and the calcium paradox: two facets of the same problem? Journal of Molecular Cell Cardiology, 10: 641-668.

Herp, A., Harris, M. and Richards, T. (1971). Correlation between the autoxidation of forcus ions or of L-ascorbic acid and the depolymerixation of hyaluronate. Carbohydrate Research 6: 395-407.

Hess, M.L. and Manson, N.H. (1984). Molecular oxygen: Friend and foe, the role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. Journal of Molecular Cell Cardiology, 16: 969-985.

Hess, M.L., Rowe, G.T., Caplan, M., Romson, J.L. and Lucchesi, B. (1985). Identification of hydrogen peroxide and hydroxyl radical as mediators of leukocyte-induced myocardial dysfunction. Limitation of infarct size with neutrophil inhibition and depletion. Advance Myocardiology, 5: 159-175.

Hohl, C., Ansel, A. Altshuld, R. Brierley, G.P. (1982). Contracture of isolated rat heart cells on anaerobic to aerobic transition. American Journal of Physiology, 242: H1022-H1030.

Hort, W. (1965). Ventrikeldilatation und muskelfaserdehnung als fruheste morpologische befunde beim herginfarkt. Virchows Arch Pathol Anat., 339: 72-82.

Hudson, R.E.B. (1965). Cardiovascular pathology. Volume 1. Edward Arnold Publishers Ltd, London. pp 649-699.

Jennings, R.B., Wartman, W.B. and Zudyk, Z.E. (1957). Production of an area of homogeneous myocardial infarction in the dog. American Medical Association Archives of Pathology 63: 580-585.

Jorgensen, L., Howsell, H.C., Having, T., Glynn, M.F. and Mustard, J.F. (1967). Adenosine diphosphate-induced platelet aggregation and myocardial infarction in swine. Laboratory Investigation, 17: 617-644.

Joseph, C.F. and Ward, P.A. (1982). Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. American Journal of Pathology, 107: 397-418.

Julian, D.G. (1978). Cardiology. 3rd. Ed. Concise Medical Textbooks, Bailliere Tindall, London, pp 144-151.

Kaplow, L.S. (1965). Simplified myeloperoxidase stain using benzidine dihydrochloride. Blood, 26: 215-219.

Kellogg, E.W. and Fridovich, I. (1975). Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. Journal of Biological Chemistry, 250: 8812-8817.

Kellogg, E.W. and Fridovich, I. (1977). Liposome oxidation and erythrocyte lysis by enzymatical generated superoxide and hydrogen peroxide. Journal of Biological Chemistry, 252: 6721-6728.

Kersten, U. (1968). Klinische untersuchurrgen am herzkranken hund. Thesis. Hannover, Germany.

Klebanoff, S.J. and Clark, R.A. (1978). The neutrophil. Function and clinical disorders. North-Holland Publishing Co, Amsterdam, Elsevier.

Klebanoff, S.J. (1980). Oxygen metabolism and the toxic properties of phagocytes. Annal of Internal Medicine, 93: 480.

Leathan, A. (1977). An introduction to the examination of the cardiovascular system. Oxford University Press, New York Toronto, pp 1-52.

Lippi, U., Cappelletti, P. and Schinella, M. (1983). Ideficit di mieloperosidasi. Piccin Editore, Padova.

Lippi, U. Cappelletti, P. and Schinella, M. (1984). Catalytic activity of neutrophil and eosinophil peroxidase. Cytochemical and biochemical study in total hereditary myeloperoxidase deficiency. Nouv Rev Fr Haematology, 26: 35-38.

Mallory, G.Z., White, P.D. and Salcedo-Salgar, J. (1939). The speed of healing of myocardial infarction: a study of the pathologic anatomy in seventy-two cases. American Heart Journal, 18: 642-671.

McCord,J.M. and Fridovich, I. (1968). The reduction of cytochrome C by milk xanthine oxidase. Journal of Biological Chemistry, 243: 5753-5760.

McCord, J.M. (1974). Free radicals and inflammation: Protection of synovial fluid by superoxide dismutase: Science, 185: 529-531.

McCord, J.M. (1985). Oxygen derived free radicals in post-ischemic tissue injury. New England Journal of Medicine, 312: 159-163.

Mead, J.F. (1976). Free radical mechanisms of lipid damage and consequences for cellular membranes. In: Free radicals in biology, Volume 1. (ed) Pryor, W. Academic Press, New York, pp 51-67.

Morgan, A.R., Cone, R.L. and Elgert, T.M. (1976). The mechanism of DNA strand breakage by vitamine C and superoxide and the protective roles of catalase and superoxide dismutase. Nucleic acid Research, 3: 1139-1149.

Mullane, K.M., Kraemer, R. and Smith, B. (1985). Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. Journal of Pharmacological Methods, 14: 157-167.

Myron, L.W. (1987). Reperfusion and reperfusion injury. Clinical Research, 35: 13-20.

Nayler, W.G., Ferrari, R., Williams, A. (1980). Protective effect of pretreatment with verapamil, nifedipine and propanolol on mitochondrial function in the ischemic and reperfused myocardium. American Journal of Cardiology, 46: 242-248.

Ohmori, H., Komoriya, K., Azuma, A., Kurozumi, S. and Oto, Y.H. (1979). Xanthine oxidase-induced histamine release from isolated rat peritoneal mast cells: Involvement of hydrogen peroxide. Biochemical pharmacology, 28: 233-234.

Paul, D.B., Henry, L. and Karl, M. (1960). The enzymes. Volume 3. 2nd Ed. Academic Press, New York and London, pp 321-322.

Paul, D.B., Henry, L. and Karl, M. (1963). The enzymes Volume 8. 2nd Ed. Academic Press, New York and London, pp 240-241.

Pigman, W., Rizul, S. and Holley, H.L. (1961). Depolymerixation of hyaluronic acid by the ORD reaction. Arthritis Rheum, 4: 240-252.

Rahamathulla, P.M. and Ashraf, M. (1984). Heterogeneity of cells response to early anoxia and reoxygenation in rat heart. Experimental Pathology, 25: 139-146.

Rahamathulla, P.M., Watanabe, K. and Millard, R.W. (1985). Prevention of lactate production and myocyte injury in isolated rat hearts perfused with perfluorochemical emulsion. Experimental Pathology, 28: 157-165.

Rechnagel, R.O., Glende, J.R., and Hruszkewycz, A.M. (1977). Chemical tetrachloride toxicity. In: Free radicals in biology Volume 3. (ed.) Pryor, W. Academic Press, New York, pp 97-132.

Romson, J.L., Hook, B.G., Rigat, H.V.H., Schork, M.A., Swanson, D.P. and Lucchesi, B.R. (1982). The effect of ibuprofen on accumulation of indium-111-labeled platelets and leukocytes in experimental myocardial infarction. Circulation, 66: 1002-1011.

Romson, J.L., Hook, B.G., Kunkel, S.L., Abrams, G.D., Schork, M.A. and Lucchesi, B.R. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation, 67: 1016-1023.

Romson, J.L., Jolly, S.R. and Lucchesi, B.R. (1984). Protection of ischemic myocardium by pharmacologic manupulation of leukocyte function. Cardiovascular Reviews, 5: 690-709.

Roos, D. (1980). The metabolic response to phagocytosis. In: Handbook of inflammation (ed.) Weissman, G. New York, Elsevier/ North Holland, pp 337.

Root, R.K. and Metcalf, J.A. (1977). H_2O_2 release from human granulocytes during phagocytosis: relationship to superoxide anion formation and cellular catabolism of H_2O_2: Studies with normal and cytochalasin B-treated cells. Journal of Clinical Investigation, 60: 1266.

Root, R.K. and Cohen, M.S. (1981). The microbicidal mechanisms of human neutrophils and eosinophils. Rev. Infect. Dis., 3: 565.

Rouser, G., Nelson, G.J., Fleicher, S. and Simon, G. (1968). Lipid composition of animal cell membranes, organelles and organs. In: Biological membranes physical fact and function, Volume 1. (ed) Chapman, D. Academic Press, New York, pp 5-69.

Sommers, H.B. and Jennings, R.B. (1964). Experimental acute myocardial infarction: histologic and histochemical studies of early myocardial infarcts induced by temporary or permanent occlusion of coronary artery. Laboratory Investigation, 13: 1491-1503.

Sydney, E.S., Cline, M.J., Schultz, J. and Lehrer, R.I.(1970). Myeloperoxidase deficiency. Immunological study of a genetic leukocyte defect. New England Journal of Medicine, 282: 250-253.

Taylor, D.H. and Sittnikow, K.L. (1968). The diagnosis of canine cardiac diseases. Journal of Small Animal Practice, 9: 589.

Thomas, M.J., Mohl, K.S. and Pryor, W.A. (1978). The role of superoxide anion in the xanthine oxidase-induced autoxidation of linoleic acid. Biochemical and Biophysical Research Communication, 83: 927-932.

Ward, P.A., Hugli, J.F. and Chenoweth, D. (1979). Complement and chemotaxis. chemical mesengers of the inflammatory process. (ed.) Houck, J.C. Biomedical Press, Amsterdam, Elsevier/North Holland, pp 153-178.

Ward, P.A. (1980). Chemotaxis, textbook of immunology. Volume 1. (ed.) Parker, C.W., W.B. Saunders, Philadelphia, pp 272-297.

Watts, J.A., Koch, C.D., and LaNoue, K. (1980). Effects of Ca^{2+} antagonism on energy metabolism: Ca^{2+} and heart function after ischemia. American Journal of Physiology, 238: H909-H916.

Weiss, S.J. and Lobuglio, A.F. (1982). Biology of disease. Phagocyte generated oxygen metabolites and cellular injury. Laboratory Investigation, 47: 5-18.

Weissman, G., Smolen, J. and Korchak, H. (1980). Release of inflammatory mediators from stimulated neutrophils. New England Journal of Medicine, 303: 27.

Willson, R.L. (1979). Hydroxyl radicals and biological damage in what relavant in vivo? Ciba Foundation Symposium, 65: 19.

Zimmerman, A.N.E. and Hulsmann, W.C. (1966). Paradoxical influence of calcium ions on the permiability of the cell membranes of the isolated rat heart. Nature, 211: 646-647.