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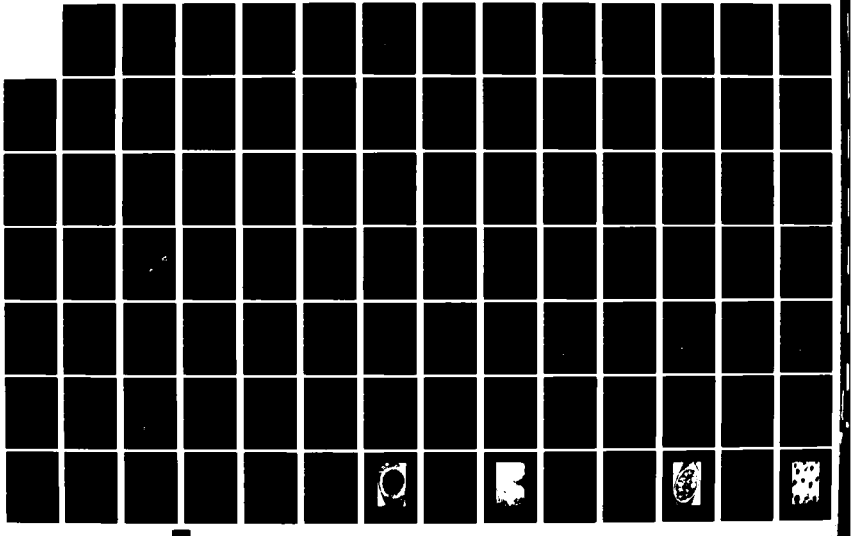

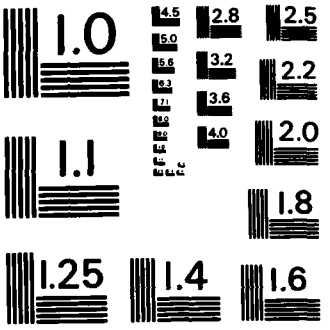

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

Degree Doctor of Philosophy Major Subject Pathology

Name of Candidate Harold Davis

Title Regional Myocardial Blood Flow and Ultrastructure Following Acute Temporary Ischemia

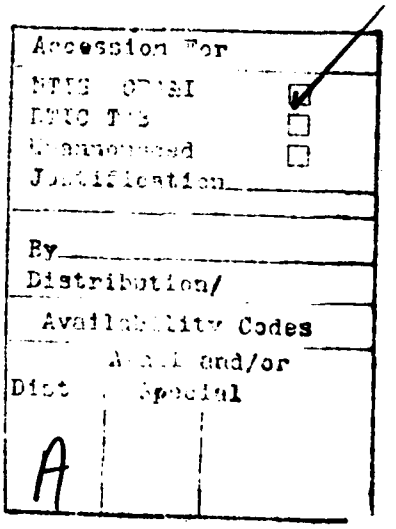
The effect of reopening acutely occluded coronary arteries has on regional myocardial blood flow and ultrastructure was studied in open chest dogs. Temporary and permanent ischemia were produced in the same animal by ligating a branch of the left anterior descending and left circumflex coronary arteries for 30 (N=6), 60 (N=6), 90 (N=5) and 120 minutes (N=6). Temporarily ischemic areas were allowed 30 minutes of reflow. Radioactive microspheres were used to measure regional myocardial blood flow. Ischemia was graded as severe (≤25%), moderate (>25≤50%), and mild (>50% of control flow).

Release of the temporary occlusion after 30 minutes of ischemia was followed by an immediate reactive hyperemia (IRH) >150% of control flow. Sixty minutes of severe temporary ischemia was followed by an IRH ≤150% of control flow in 16 of 36 samples. Eleven of these 16 samples had an IRH ≤60% of control flow. An IRH ≤150% of control flow occurred in 8 of 32 and 9 of 37 samples following 90 and 120 minutes of severe ischemia, respectively. In all groups, reflow inhibition had disappeared 30 minutes post-release in all but one sample.

Ultrastructural alterations (i.e. myocyte swelling, glycogen depletion, nuclear chromatin margination and mitochondrial swelling) produced with 30 minutes of ischemia were reversed with reflow. Conversely, reflow intensified lesions after 60-120 minutes of severe ischemia. These changes included contraction bands, mitochondrial fragmentation and sometimes intramitochondrial

inclusions. Focal to diffuse endothelial cell swelling, membrane blebbing, and widened cell junctions were present in less than 10% of the capillaries and venules after 120 minutes of ischemia. These lesions were not affected by reflow and did not correlate with tissue IRH.

Reflow was most markedly inhibited in severely ischemic myocardium after 60 minutes of ischemia; reflow was less inhibited after 90 or 120 minutes of ischemia. Peak vascular alterations occurred after 120 minutes of ischemia and thus, were not felt to be primarily responsible for the observed reflow inhibition. Vascular spasm was considered as an alternative mechanism responsible for this phenomenon, possibly as the result of a humoral agent present in ischemic myocardium acting on contractile vessels.



Abstract Approved by: Committee Chairman Sanford P. Bishop

Program Director Sanford P. Bishop

Date May 17, 1982 Dean of Graduate School

REGIONAL MYOCARDIAL BLOOD FLOW AND ULTRASTRUCTURE FOLLOWING ACUTE TEMPORARY ISCHEMIA

by

Harold Davis

A dissertation

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Pathology
in The Graduate School, University of Alabama in Birmingham

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1982

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Date

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Accomplishments and milestones are rarely the achievement of a single individual. This dissertation is further proof of this truism. I could not have begun to complete a project of this magnitude without the steady, unfailing guidance of an advisor who was well aware and empathetic of my personal problems and knowledgeable of the subject with which this project dealt. Because of the challenges Dr. Sanford P. Bishop set before me and the environment he created in which I might work, I shall forever consider him my mentor and my friend. Special thanks are also extended to Ann Rose. Her knowledge of basic computer programming and technical expertise in the use of radioactive microspheres were invaluable. The photographical expertise of Danny Musick, and the surgical aid provided by Ann, Danny and Barbara Winch were also very important in the completion of this project. I consider it an honor and a privilege to have matriculated at the University of Alabama in Birmingham in the midst of those mentioned above and numerous others that are not.

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CHAPTER I

REVIEW OF LITERATURE

Introduction

At its most fundamental level, myocardial ischemia, like ischemia in other organs, has as its primary cause an imbalance between oxygen supply and oxygen demand. The factors leading to this imbalance are often multiple. Hill (49) in a study of cerebral ischemia has shown that there are basically two factors determining the outcome of temporary ischemia: 1) whether or not parenchymal cells survive the ischemia, and, 2) whether following the end of ischemia normal flow is resumed and maintained. These two determinants, one would logically deduce, are not peculiar to cerebral ischemia. It has been eloquently shown by numerous investigators (18,57,63,117) that cellular damage during and following ischemia exists as a continuum. Some cells are irreversibly injured while others suffer damage that is at least potentially reversible. Some cardiovascular researchers have serendipitously found ultrastructural morphological ischemic changes within the coronary microcirculatory system (63,65,66,85,117). These changes have been well defined for the microcirculatory systems of the kidney, skin and brain (3,27,108,120). Recent studies dealing specifically with these changes in the coronary microvasculature have been reported (4,22,42,64,116). These changes, to be discussed later, are thought to interfere directly or indirectly with the reinstitution of flow within these vessels following the termination of ischemia of some "critical" time span. These changes are thought to be very important in the genesis of the "no-reflow" phenomenon.

Objective and Specific Aims

The objectives of this study were to evaluate the temporal and structural relationships of ischemia induced injury in the dog myocardium.

To accomplish this objective there were four specific aims:

1. To evaluate alterations in regional myocardial blood flow in areas of acute ischemia following revascularization, with emphasis on identification of the no-reflow phenomenon.

2. To define the normal ultrastructure of the canine coronary microcirculatory system and to compare these findings with similar published data available for man.

3. To study the changes in ultrastructural morphology that occur within the canine coronary microcirculatory system due to varying periods of acute ischemia.

4. To evaluate the relationship of these morphologic changes to the pathogenesis of the "no-reflow" phenomenon.

Background

In 1976 ischemic heart disease caused approximately 650,000 deaths in the U.S., roughly 33% of the total 1.9 million deaths for that year (96,106). Though the mortality rate for ischemic heart disease has declined steadily since 1970 following its peak incidence in the mid-1960's, it continues to be an important disease (96,106). Ninety percent of all cardiac deaths are due to ischemic heart disease and it causes more deaths than all forms of cancer and infections combined (96). There is little wonder then that there has been a copious amount of literature written on the subject of ischemic heart disease within the last ten years. Few authors have addressed the issue of what defines the normal coronary microcirculatory system ultrastructurally (105,112) or its relationship to ischemia even though we know from our experience with the kidney and other organs how important this can be. Tilmanns et al. (112) deals more with red cell velocity

within capillaries as opposed to the structure of capillaries. Only the work by Sherf et al. (105) deal primarily with the normal ultrastructure of the coronary microcirulatory system. Their work was done using biopsy samples of human hearts from patients undergoing heart surgery for a variety of reasons. Their biopsies were taken from areas of myocardium considered normal for each individual patient. Dogs have been used as one of the major animal models for studying myocardial ischemia; however, there is no detailed study designed to compare the ultrastructure of the human and canine coronary microcirculatory systems. The microcirculations of the dog and man have correctly or incorrectly, definitely unscientifically, been assumed to be comparable to the extent that any differences are considered functionally insignificant. Physiologists have long known that this is the area of important gas and nutrient exchange. Our knowledge, or the lack of it, about this system has perhaps inhibited a clearer understanding of its function during normal and abnormal states. The components of this system include arterioles, precapillary sphincters, metarterioles, capillaries and venules. The size of these vessels is thought to vary tremendously depending on whether the tissue was immersion or perfusion fixed and at what pressure they were perfused. Therefore, the size of these vessels should serve only as a loose criterion for identification.

Sherf et al. (105) used the criteria of Rhodin (93,94) and others (12,34,35,77,78) to define the components of this system. It is important to remember that they and Rhodin used nonperfusion fixed tissue. Rhodin's tissues (93,94) were fixed in situ by dropping fixative on them. Sherf et al. (105) described arterioles as the smallest distributing vessels in the body. They are 30-100 μm in diameter and are lined by thin elongated endothelial cells (50 μm x 2 μm at the nucleus). Their nuclei are flat and long (8 μm x 2 μm) and they do not protrude into the lumen. These cells have a basement membrane and elastic

fibrils can be seen between it and the media. The media consist of 2-3 continuous smooth muscle layers arranged in parallel circular layers. These cells, like the endothelial cells lining the lumen, have numerous pinocytotic vesicles in their cytoplasm and they have a basement membrane.

Precapillary sphincters regulate local flow in the capillary bed and they are also responsible for the periodic flow and the low pressure in capillaries (112). Honig (53) using microcinematography suggested that these sphincters are found in the heart as they had been predicted to occur in other tissues. Schaper and Schaper (99) have questioned the significance of these vessels and have even questioned their exsistence in the heart. They proposed the intermittent flow in some capillaries could be the result of arteriole activity.

Two hypotheses have been proposed to describe precapillary sphincter function (47,67,97,121,124). The first one is the capillary-pressure hypothesis. Harris and Longnecker (47) have proposed that precapillary sphincter activity might serve to regulate the rate of tissue fluid exchange by controlling the exchange of water between the intravascular and interstitial compartments. If the sphincters are relaxed, hydrostatic pressure throughout the local capillary bed would rise and filtration would predominate. Contraction of these sphincters would cause hydrostatic pressure to fall and the rate of passage through capillaries would decrease, and therefore, reabsorption would predominate. This ability of local circulation to respond to a pressure gradient was first suggested by Roy and Brown (97) in 1879.

The second hypothesis is the tissue-metabolism hypothesis first suggested by Krogh (67). Zweifach (124) and Harris and Longnecker (47) offered modifications to this hypothesis. This hypothesis stated that sphincter relaxation allows for an increase in the exchange of metabolites and waste by increasing the total surface area across which exchange can occur. This results from the increased time each

capillary has flow and the increased numbers of capillaries with flow. Sphincter contraction reduces the total surface area for exchange by decreasing the amount of time for exchange and by reducing the number of vessels with flow, thus substrate depletion and waste accumulation occurs.

Honig (53) used Rhodin's EM micrographs (93) to propose a sphincter reaction model, a variation of the tissue metabolism hypothesis, dependent on oxygen supply. In this model relaxed sphincters receive their oxygen supply directly from arterial blood and during contraction they get it from the interstitial space. Therefore the duration of sphincter contraction would be based on tissue PO_2 and the duration of relaxation on arterial PO_2. Harris and Longnecker (47) agreed with Honig and suggested that reduced arterial PO_2 increased the duration of flow within a capillary by causing sphincter relaxation. They also felt that during exercise the reduction in tissue PO_2 increased the duration of flow because the low tissue PO_2 decreased the duration of sphincter contraction. They state that in tissues with a low metabolic rate the regulation of capillary pressure may be the major function of these sphincters while in tissues with a high metabolic rate, i.e., the heart, the regulation of wastes and substrates may be most important. Berne (13), Berne and Rubino (14), and Schaper and Schaper (99) and others have suggested a relationship between vascular tone and adenosine. Adenosine is a potent vasodilator produced as a by-product of ATP metabolism. It can leave myocytes and theoretically affect nearby small vessels. This would include precapillary sphincters. Adenosine may be acting alone or with oxygen as proposed by Honig (53) and Harris and Longnecker (47).

Sherf et al. (105) define precapillary sphincters as vessels that are 30-15 μm in size. The short broad endotheial cells lining them average 5 μm in length and 4 μm in width at the nucleus. The nuclei of these cells are round to oval, unlike the flat nuclei of arterioles. These nuclei are large, approximately 4 μm x 3 1/2 μm,

and bulge into the lumen of these vessels. The nuclei frequently bulge into the lumen so dramatically that in some non-perfusion fixed material they seem to occlude these vessels. The media of these vessels is composed of a single continuous layer of smooth muscle cells. Cytoplasmic projections from smooth muscle cells connect with projections from endothelial cells. Rhodin (93) called these projections myoepithelial bridges. These bridges are numerous and finger-like with a tight membrane to membrane junction (105). They are surrounded by a basement membrane that is continuous with the basement membrane of smooth muscle cells and endothelial cells. The bridges illustrated in Rhodin's paper (93) are broad, short and stump-like rather than finger-like as described by Sherf et al. (105). Rhodin's material was from the medial thigh of rabbits and was fixed in situ by dripping 1% osmium or 2% glutaraldehyde on the exposed area. Fawcett (33), Rhodin (93,94) and Sherf et al. (105) have all suggested that these bridges might aid in the rapid deliverance of vasoactive substances, originating within the lumen, to smooth muscle cells in the wall.

The portion of the capillary bed that receives continuous rather than intermittent flow is called the central or thoroughfare channel. At the proximal end of this bed just distal to the arteriole is the metarteriole. These vessels range from 10-20 μm in size (105). They have a sponge-like endothelial cell layer with cytoplasmic villi projecting into the lumen and large cytoplasmic vacuoles. The nuclei of these cells bulge into the lumen much like those in precapillary sphincters. The media is composed of a single incomplete smooth muscle layer. In the larger metarterioles myoepithelial bridges are present.

Capillaries range from 8-5 μm in size (105). They are lined by one to three endothelial cells that usually have villous projections, cytoplasmic vacuoles and pinocytotic vesicles as in metarterioles. Endothelial flaps can easily be seen at the endothelial cell junctions. These flaps are the result of one cell overlapping

another. The nucleus in each endothelial cell is still quite large but instead of bulging into the lumen it bulges toward the extracellular space. These vessels have no muscular layer and few collagen fibers surround them. Pericytes are often seen in close proximity. Caulfield (25) beileves that there is considerable collagen around these vessels and that it is functionally important. He states that the amount of collagen surrounding these vessels is only small when compared to the amount surrounding other vessels. According to Caulfield (25), the scanning electron microscope allows a more accurate interpretation of the amount of collagen surrounding these vessels. He has also suggested an active role for this collagen in keeping capillaries open during systole. Sherf et al. (105) did not report the presence of a significant amount of collagen surrounding capillaries.

Venules range from 15-100 μm in size and are the most distal component of the microcirculatory system (105). They have thin, long endothelial cells similar to those lining capillaries. Their nuclei are long and flat (7x2-1/2 μm) and their cytoplasm has fewer vacuoles and pinocytotic vesicles than capillary endothelium. Larger venules may have poorly developed smooth muscle within their wall. Characteristically, venules have a large amount of collagen surrounding them and it often seems to be anchored at the basement membrane of their endothelial cells. Contrary to the idea of Caulfield (25), Sherf et al. (105) have proposed that capillary-like vessels with a large amount of collagen in their walls should be classified as venules.

Schaper (100) has previously defined differences in the collateral circulation between man and other animals. He has found that collaterals in the normal human heart are few in number and are located subendocardially as are those in the pig. Collaterals in the dog were located primarily subepicardially. In this same reference he also discusses the difference in the ability of man, dog and pig in developing collaterals. He states that the pig cannot develop epicardial

collaterals, the dog can develop endocardial collaterals to a limited degree and man can develop collaterals throughout the heart. Though man develops collaterals preferentially in the subendocardial zone, large epicardial collaterals are common in diseased hearts. These types of differences are very important to know if data derived from animal studies are to be intelligently extrapolated to man. If the dog is to be used as an animal model for human myocardial ischemia, then it is important to identify morphological similarities and differences in the coronary microvasculature of these species.

Effects of Ischemia

The microcirculation, like the tissue it services, requires a fairly steady source of nutrients and the removal of waste. Ischemia, therefore, can injure these vessels. The changes that occur in these vessels during and following ischemia should have a profound effect on the viability of the parenchyma supplied by these vessels. The ischemic changes in the morphology of these vessels should occur as a continuum that is related to the duration of the ischemia. The earliest change appreciable at the EM level is the occurrence of edema or swelling of mitochondria and/or the entire endothelial cell (4,42,64). This change can be seen after only 10-40 minutes of ischemia, though even after 1 hour of ischemia some cells at risk will retain their normal volume (64). This is a consistent early change also seen in cutaneous (120), cerebral (3,27) and renal ischemia (41). Ischemic degenerative changes, i.e., decreased pinocytotic vesicles, nuclear chromatin clumping, and dilated endoplasmic reticulum have also been reported to occur (3,27,39,42,64). Kloner, Ganote and Jennings (64) have reported that after 90 minutes of ischemia multifocal separations of endothelial cells occur, thus exposing the basal lamina. Armiger and Gavin (4) made similar observations at 120 minutes of ischemia. Large intraluminal clear blebs were seen by Kloner et al. (64) at 90 minutes of ischemia. These blebs, according to Armiger

and Gavin (4), along with cell debris and RBCs filled the lumen of most vessels at risk after 120 minutes of ischemia. Kloner et al. (64) have reported that subsarcolemmal blebs, the result of intracellular myocyte edema, along with focal fibrin clumps appeared at times to occlude the capillary lumen following 90 minutes of ischemia. Ames et al. (3) state that similar changes in rabbit brains occur simultaneously with the period of time necessary for ischemia to cause death. After only 5 minutes of ischemia they found capillaries occluded due to swollen perivascular astrocytes (3). However, in a subsequent report, this group reassessed their earlier findings on cerebral ischemia and vascular injury (37). They reported that severe perivascular astrocyte edema and subsequent capillary occlusion following cerebral ischemia did not occur. Furthermore, in ischemia lasting up to 30 minutes they did not find endothelial cell swelling or blebing in capillaries. This contradicts not only their earlier work (3) but that of Chiang et al. (27) as well. Fischer et al. (37) attributed this difference to either the use of different perfusion fixation techniques or because blood cells were removed from their brain samples prior to the induction of ischemia. They state that the difference in fixation procedures is probably the more important.

Numerous investigators (2,43,46,110) have reported the beneficial effects of glass-wool filtration of whole blood before perfusing post-ischemic tissue. These studies involved the brain or kidneys of dogs and cats, and suggest some element present in whole blood, but not present in filtered blood may serve to further damage ischemic tissue upon reperfusion. The writer will present data later that demonstrate capillary blebs and endothelial edema in capillaries of permanently occluded immersion fixed ischemic myocardium. The writer does not think that perfusion fixation techniques alone can account for the difference between Fischer and his associates (37) data and that previously reported by others. Wilms-Kretschmer and Manjo (120) have reported similar endothelial blebs in the

skin, but felt that even after 2 hours of ischemia these blebs did not completely occlude these vessels. An occlusion of around 70-90% may be enough, however, to cause functional stenosis of these vessels. The investigators quoted above have dealt with ischemic changes occurring in capillaries. None has mentioned if any changes occur in the other components of this system.

Reflow after Ischemia

Coronary bypass surgery, angioplasty and fibrinolysis treatment have made the reestablishment of blood flow to ischemic myocardium a reality. It is important to know when these heroic efforts will result in the restoration of blood flow to salvageable tissue and when, if ever, it makes a bad situation worse. Several investigators (43,92) have demonstrated beneficial effects including infarct size reduction, from reperfusion following varying periods of ischemia. However, other investigators have reported detrimental effects associated with reperfusion (19,52) The work of Costantini et al. (29) has contributed a great deal to the clarification of this paradox. They used two groups of dogs in which myocardial ischemia had been induced. One group had been subjected to ischemia lasting 7 days and the other to 3 hours of ischemia followed by 7 days of reperfusion. The permanently occluded group demonstrated depressed mechanical and metabolic functions throughout the 7 days of the study. Their infarcts averaged 32% of the left ventricle. The mechanical function and metabolic state of the reperfusion group was initially depressed and did not improve immediately with reflow. In fact, there was a tendency for them to worsen initially. Seven days following the initiation of reperfusion they showed significant improvement, and infarct size averaged only 14% of the left ventricle. Their study indicates that reperfusion following an acute ischemia episode causes a period of depressed unstable mechanical and metabolic status. This period is characterized by

hemorrhage, arrhythmias, edema and enzyme loss (29,52). Following this initial period, a return toward normal function seems possible since reperfusion may serve to salvage viable but damaged cells.

Hoffmann et al. (52) state that hemorrhage will result if ischemia lasting six hours or longer is followed by reperfusion, but like reperfusion following three hours of ischemia, it does not increase infarct size. Fishbein et al. (38) have reported that when hemorrhage occurs following myocardial ischemia and subsequent reperfusion, it is limited to tissue already necrotic. Hemorrhage, therefore, is the result of prior damage and does not seem to cause further damage. However, they state that the effect of hemorrhage on healing and possible aneurysm formation cannot be determined at this time.

No-reflow Phenomenon

A phenomenon called the no-reflow phenomenon has been reported to occur in the kidney, skin, brain, and heart of various mammals (3,21,27,58,64,108,120). The essence of this phenomenon is the failure of normal flow to resume at the microvascular level even after the end of vessel occlusion, following ischemia of a critical duration. Krug, de Roachemont and Korb (68) and Willerson et al. (119) were among the first investigators to suggest that this phenomenon occurred in the heart. It was Kloner et al. (64) who first studied ischemic changes at the EM level in the coronary microvasculature and then associated these changes with this phenomenon. They subjected dogs to permanent or temporary ischemia for 40 or 90 minutes. Intravenously injected carbon black or thioflavin-S (a fluorescent vital stain for endothelium) was used to check for successful reperfusion in the temporary ischemia model. In hearts reperfused after 40 minutes of ischemia, long enough to cause some irreversible myocyte damage, the dye and the carbon black were uniformly distributed. This meant that any morphological changes in the microvasculature were not significantly restricting the flow of carbon

particles or the dye. After 90 minutes of ischemia, only the outer half of the portion of the ventricular wall at risk was penetrated by the tracers. The failure of flow restoration was associated with capillary damage including endothelial bleb formation and focal swelling.

Kloner et al. (64) noted that death to myocytes occurred before severe capillary damage was observed and before the no-reflow phenomenon began. This phenomenon, once it occurred, seemed limited to the subendocardium. Kloner et al. (64) did not address the issue of whether longer periods of ischemia might create a similar pattern in the subepicardium. Even after 90 minutes of ischemia there is some salvageable tissue in the inner one third of the myocardial wall; however, the no-reflow phenomenon could make salvage impossible.

Camilleri and Fabiani (21) and Sunamori et al. (109) reported that the no-reflow phenomenon can occur in dogs on cardiopulmonary bypass. They have suggested that this phenomenon is responsible for the occasional subendocardial ischemia seen in these dogs and in man following removal from these machines. Flores et al. (39), Franklin, Ganote and Jennings (41) and Summers and Jamison (108) have proposed that this phenomenon might potentiate renal ischemia lesions by preventing the salvage of viable but injuried nephrons. Chiang et al. (27) and Ames et al. (3) have suggested the same is true in the brain. May et al. (74), Chait et al. (26) and Wilms-Kretschmer and Manjo (120) have also demonstrated similar findings in surgically prepared experimental cutaneous free flaps. Kloner et al. (64) and Darsee and Kloner (30) have reported that in the heart this is unlikely to be true. They found the no-reflow phenomenon only in the central necrotic core of myocardial infarcts. Therefore, the no-reflow phenomenon could not possibly inhibit or prevent reperfusion to reversibly damaged myocytes. The findings of White, Sanders and Bloor (118) add credence to Darsee and Kloner's (30) proposal. In their work, they report that ligating the left circumflex coronary artery of dogs

for 2-6 hours followed by 3-30 hours of reflow resulted in no-reflow in only .5-1.5g of tissue. They also found that no-reflow was limited to the central core of the infarct.

Various causes of the no-reflow phenomenon have been suggested by numerous investigators (3,21,27,39,58,74,108,120). Kloner et al. (64) have attempted to summarize them. 1) Endothelial protrusions (blebs) seen in capillary lumens may play an integral part in limiting the reinstitution of flow. 2) Endothelial cell swelling may hinder flow. 3) Red blood cells frequently appear packed in capillary lumen and this local increase in hematocrit could lead to an increase in viscosity that might affect flow. 4) Thrombosis, especially in those areas where endothelial cells have separated and exposed the basal lamina. 5) Myocyte edema is often severe and subsarcolemmal blebs appear to occlude some capillaries.

Each of these possibilities carry with it serious questions about its validity. Lumenal blebs rarely seem to be totally occlusive (64). The same is true for swollen endothelial cells (64). Investigators have reported contradicting findings concerning the important of cell swelling and this phenomenon. Chiang et al. (27) have reported diffuse endothelial swelling in capillaries of ischemic cerebral tissue but Kloner et al. (64) and Gavin et al. (42) have reported a patchy often partial occlusion of capillaries in the dog heart. Chait et al. (26) have shown that perfusion of ischemic cutaneous free flaps with hyperosmolar solutions had no beneficial effect on skin survival. Flores et al. (39) have reported that infusing hypertonic mannitol corrects cell swelling, delays the onset of the no-reflow phenomenon and also delays renal dysfunction following renal ischemia. Equivalent volumes of isotonic saline or isotonic mannitol were not beneficial. Willerson et al. (119) and Powell et al. (87) have shown beneficial results with hypertonic mannitol given before or after induced myocardial ischemia in the dog.

Parker et al. (83) reported that continued reperfusion following two hours of myocardial ischemia leads to a progressive increase in coronary vascular resistance. They suggest that perfusing with hyperosmotic mannitol may be initially beneficial. However, they also state that it does not deter the progressive increase in coronary vascular resistance and the subsequent hypoperfusion of the subendocardium.

Though RBC packing in capillaries has been reported by several investigators (39,58,108); its importance in the genesis of the no-reflow phenomenon is still questioned. Leaf (69) has reported that red blood cells lose their flexibility when their ATP levels are low. This flexibility is very important since many capillaries are smaller than individual red blood cells. RBC packing has not been uniformly seen and its significance is still questionable. It is possible that these studies may be suggesting that some combination of red blood cell packing and endothelial cell swelling may be the cause of no-reflow. Earlier the writer cited references (2,43,46,110) that implied some agent in whole blood may be responsible for the detrimental effects associated with reperfusing post-ischemic tissue. The agent in these studies was not the intact red blood cells. Johnston and Latta (58) have studied the effects of blood being present in glomeruli at the time ischemia is induced. They reported more severe mesangial and endothelial cell swelling and subsequently greater inhibition of post-ischemia reperfusion. They have suggested hemoglobin released from damaged red blood cells may be toxic in the presence of hypoxia and acidosis.

Thrombosis and myocyte edema are both not yet completely accepted as being necessary to produce no-reflow. Thrombosis was not seen by Gavin et al. (42) in tissues which demonstrated no-reflow. Others (3,39,70,108) have reported this phenomenon occurring in renal and cerebral tissues of heparinized animals. Edema in perivascular myocytes and subsequent compression of capillaries have

have been reported by Kloner et al. (64) and Jennings, Baum and Herdson (56). Gavin et al. (42), however, reported they observed capillaries that were dilated rather than compressed in tissues showing the no-reflow phenomenon. Kloner et al. (64) did observe occasional myocyte compression of capillaries in tissues undergoing only 40 minutes of ischemia and failing to show no-reflow.

Other suggestions as to the cause of the no-reflow phenomenon in the heart have been made. Krug et al. (68) and Gavin et al. (42) have suggested that vascular spasm might be important. This would mean spasms in either precapillary sphincters, arterioles or both. Secondly, Caulfield (25) suggests that collagen fibers which anchor capillaries to myocytes, keep these vessels opened. During ischemia, the area affected has ceased to contract and he theorizes that failure of myocyte contraction may contribute to preventing capillary distension and causes no-reflow. Thirdly, it has recently been reported that neutrophils may cause capillary plugging and possibly no-reflow (32). Finally, Lee et al. (70) have suggested that either global or focal areas of myocardial contracture are responsible for the failure of reperfusion following ischemia. In their experiments dogs were placed on cardiopulmonary bypass and their hearts maintained at 38°C (normothermia) or 28°C (hypothermia). Ischemic arrest was induced by cross clamping the ascending aorta. Ischemia was maintained for 30-45 minutes. Hearts maintained at 38°C became severely contracted. Contraction was detected by the displacement of fluid from a balloon placed in the left ventricle. Microspheres were injected immediately after the initiation of reperfusion to determine if no-reflow had occurred. They reported that though the subendocardium region was more edematous than other areas in both groups, no-reflow developed only if myocardial contracture was present.

The writer believes the no-reflow phenomenon does occur in the heart. Either a single or combination of ischemia induced ultrastructural and functional

changes in the coronary microvasculature causes it. In the present study blood flow will be determined during control, ischemia and post-ischemia periods in order to detect the occurrence of the no-reflow phenomenon. Ultrastructural changes in the microvasculature will be studied in an attempt to decide what does cause the no-reflow phenomenon.

Previous methods of studying this phenomenon and regional blood flow in post-ischemic myocardium have been inadequate. The markers or tracers used by Kloner et al. (64), thioflavin-S and carbon black, and Gavin et al. (42), colloidal thorium hydroxide labelled with fluorscein, are not similar in size or flow characteristics to red blood cells. When used alone, these markers are incapable of adequately identifying early functional obstruction.

Microspheres

Pohlman (86) in 1909 first reported the use of solid particles to study blood circulation. He injected starch granules into fetal pigs to study the flow of blood within their hearts. Heymann et al. (50) have credited Prinzmetal et al. (88) with first using glass microspheres to detect vascular anastomoses in human hearts and in various rabbit organs. In 1970 Zolle, Rhodes and Wagner (123) reported the use of albumin macroaggregates labelled with radioactive iodine to study circulation in humans. Rudolph and Heymann (98) had three years ealier reported the use of radioactive labelled carbonized microspheres. These spheres were used to study the circulation of sheep and goat feti in utero. Numerous investigators (6,7,15,16,17,28,31,40,55,62,80,84,114,118) have recently reported the use of these spheres to study cardiac output and regional myocardial blood flow. Heymann et al. (50) have reviewed the use of these spheres. In this review they have discussed in detail the technical aspects of the use of radioactive microspheres. Some aspects of microsphere use presented in their work and in others will be discussed.

The successful use of microspheres requires that several practical assumptions be made. These include: 1) they must have rheologic characteristics similar to red blood cells, 2) adequate mixing of the spheres occur, 3) microsphere injections do not significantly affect circulatory function, and 4) practically all microspheres are trapped in the first capillary bed they perfuse.

Rudolph and Heymann (98) present data showing an excellent correlation between blood flow to the brain measured with a flowmeter and with radioactive microspheres. Utley et al. (114) demonstrated that total coronary blood flow measured with microspheres differed from results obtained with a flowmeter by under 20%. They reported a difference of less than 10% in 66% of their measurements. Buckberg et al. (120) have shown in sheep and lamb that coronary blood flow calculated with microspheres averaged 1.4% less than values obtained with flowmeters. In dogs they found an average difference of 3.6% less. When they compared renal blood flow calculated with microspheres to flowmeter derived values they found a difference of 6 ± 2% in the dog. These findings indicate microspheres do in fact have rheological characteristics similar to red blood cells.

Buckberg et al. (20) have addressed the issue of adequate mixing. They report that multiple injections into the left atrium of dogs give flow results whose differences can be explained by random error. Heymann et al. (50) have recommended spheres be injected at a site proximal to the first major arterial branch point supplying the organ of interest. Investigators studying coronary blood flow seem to prefer left atrium injections (15,16,17,50,62). Injection of microspheres into the left ventricle is used by some investigators. However, if microspheres are injected near the aortic outflow tract poor mixing may occur. The coronaries could, therefore, be perfused with microsphere poor blood. Bishop (15) reported on a series of 29 dogs from which 74 paired femoral artery reference samples were collected following left atrial microsphere injection. Ninety

percent of these samples had less than a 5% difference in microsphere number. These 29 dogs included both closed and open chest preparations; no difference was observed between the two preparations.

Microsphere injections have been shown by Heymann et al. (50), Bishop (15), and Millard, Baig and Vatner (76) to have no persistent damaging hemodynamic effect. However, Bishop (15) and Millard et al. (76) have reported the occurrence of an occasional transient drop in systemic blood pressure. Both report that this occurs primarily following the first injection of microspheres and rarely with subsequent injections. Five to eight percent of dogs and a high percentage of monkeys may demonstrate this phenomenon (15). In dogs this pressure drop averages 15-20 mm Hg and lasts approximately 30 seconds (15). This drop in pressure is due to the presence of Tween-80, a surfactant used to inhibit microsphere clumping (76). Electrocardiographs and blood pressure tracings should be obtained with each injection so that the occurrence of a transient pressure drop or more serious myocardial dysfunction can be noted.

Blood flow calculations are possible with microsphere usage because they do not recirculate to any significant degree. They are overwhelmingly trapped during their first passage through an organ. Utley et al. (114) and Buckberg et al. (20) have reported that injecting 8-10 μm microspheres into the left ventricle or atrium result in less than 1% nonentrapment of spheres within the myocardium. This value was obtained by measuring the number of spheres in coronary sinus blood samples. In their review, Heymann et al. (50) cite several studies that show there is an insignificant amount of nonentrapment in the heart and kidney when 9 or 15 μm microspheres are used.

Bishop (15) has reported that less than 0.5% nonentrapment in the dog myocardium occurs with the use of 15 μm microspheres. He also found that 9 μm spheres showed 1-4% nonentrapment. A 10 to 20% nonentrapment rate has been

reported to occur in the head of the dog with both 9 and 15 μm microspheres and thoracotomy causes a sharp decrease in this value (15). Since the amount of nonentrapment within organs will vary, an important technical aspect of microsphere use is deciding what size spheres to use.

Microspheres that are close to the same size and density of red blood cells are more likely to behave like them. If an organ has a large number of arteriovenous anastomoses more nonentrapment is likely to occur. In such organs, larger spheres should be used. Fortunately, total blood flow to several organs have already been determined along with the amount of nonentrapment for several sizes of microspheres. However, if an investigator cannot find credible data in the literature citing the amount of nonentrapment in a particular organ with the use of a specific size microsphere he needs to first generate it. Deciding what size microspheres to use is important in measuring regional as well as total organ blood flow.

Domenech et al. (31) showed that the ratio of the number of spheres per gram in the subendocardium to the number in the subepicardium is related to the size of spheres used. They found using spheres from 51-61 μm in diameter resulted in a ratio of 2.5. When 20-33 μm spheres were used a ratio of 1.4 was obtained. A ratio of 1.3 resulted when 14 μm spheres were used. Bishop and Davis (16) injected 9 μm and 15 μm microspheres simultaneously into the left atrium of closed chest anesthetized dogs. They then opened the chest of these animals and injected more 9 μm microspheres. The 15 μm microspheres resulted in a subendocardium to subepicardium blood flow ratio of 1.39. The simultaneously injected 9 μm microspheres in the closed chest preparation resulted in a ratio of 1.18 and in the open chest preparation a ratio of .93. Bell and Fox (10) have reported 40% greater blood flow to the subendocardium in the open chest dog when measured with 15 μ m microspheres.

Utley et al. (114) tested an array of sphere sizes (25μ, 15μ, 9μ and filtered 1-10μ) and judged the 9μm spheres as being best able to measure regional myocardial blood flow. The 9μm spheres agreed best with methods employing the Fick principle.

Domenech et al. (31) and Utley et al. (114) have both reported that the larger spheres overestimate subendocardial flow. They theorized that since large particles tend to travel axially when large microspheres are used, mircrosphere poor blood is skimmed off by proximal branches to the subepicardial area. Bishop and Davis (15) have demonstrated that this pattern of greater subendocardial to subepicardial blood flow is not due completely to the size of microspheres used. Thallium-201 was given intravenously to dogs after a microsphere injection and just prior to removal of the heart. Thallium-201 acts similarly to potassium in that it is rapidly taken up by cells in proportion to blood flow (107). They reported a correlation of .925 between the counts per minute seen with microspheres and those of Thallium-201 and both demonstrated a greater subendocardial flow.

A major technical question to be answered when deciding whether to use any scientific technique is: do the limitations and associated errors make the results unbelievable? Buckberg et al. (20) have evaluated several sources of error in the use of the microsphere technique to measure blood flow. Among these were the minimum number of spheres a sample must contain and the optimal duration and method of reference sample collection. Also studied was the difference between left atrium and left ventricle injections and the accuracy and limitations in measuring organ flow with this method. They found when their samples contained at least 400 spheres the difference in flow for similar size spheres was under 20%. This error or difference was attributed to some nonrandomness of sphere distribution resulting from poor mixing. The number of spheres present in a sample was found to be more important than their total activity. Low activity

could be compensated for by longer counting times. However, the smaller the number of spheres present in a sample the higher the probability that a nonrandom distribution occurs. Increasing the counting time will not correct for this problem.

Use of the microsphere technique requires at least one reference sample of arterial blood be withdrawn at a known rate and for a specific time interval with each injection. The number of microspheres present in this volume of blood obtained at a known flow rate is used to compute blood flow to the tissue being studied. The reference sample should be drawn at a sufficient rate to insure 400 spheres are in each sample (20). This is related not only to the rate and length of withdrawal times but also to the number of spheres injected.

The length of withdrawal time will also depend on the size of the animal, and the size of the catheter used. Studies done in our laboratory have shown that the withdrawal catheter should have a dead space that does not exceed one half the flow rate through the system. For example, a 10 ml per minute flow rate should be withdrawn using a catheter whose dead space is no larger than 5 ml. This ensures that all microspheres are cleared from the catheter when a withdrawal time of three minutes is used. Use of catheters with a larger dead space to flow ratio result in incomplete microsphere clearance from the catheter and an underestimation of true tissue blood flow.

Several investigators have studied the possible loss of preocclusion injected microspheres from infarcted myocardial tissue (24,60,71,79). Such a loss would result in a systematic underestimation of control blood flow. Capurro et al. (24) were among the first to report microspheres were lost from ischemic myocardium. They reported 30% of 15 μm microspheres in the dog were lost from ischemic myocardium within 24 hours of occlusion. They suggested this technique was unsuitable for quantitative assessment of coronary collateral flow when the time between injection, occlusion and tissue collection was greater than 24 hours.

Jugdutt et al. (60) reported 19% of 7-10 μm microspheres were loss from necrotic myocardium in the dog. They found a correlation of .93 between the extent of necrosis and the degree of microsphere loss. In their samples microsphere loss occurred only when the amount of histologically determined necrosis was 37% of the area at risk or greater. When they killed dogs within six hours of preocclusion microsphere injection no microsphere loss was seen. Desiccation of their samples showed that 40% of their apparent 19% total loss could be attributed to tissue edema, resulting in increased tissue weight. An excess of microspheres were found in the lungs and neck lymph nodes in their dogs with necrosis but not in dogs without necrosis. They suggest there may be a greater subepicardial loss of microspheres, when this area is involved, because of more collaterals in this area. Lekven and Andersen (71) reported an increase in the number of 15 μm microspheres present in the lungs of cats 10 hours after a preocclusion left atrial injection. This increase in microspheres in the lung was associated with a 16% average loss from ischemic myocardium. They also reported a 6% and 8% loss in two cats 3 hours after occlusion and suggest necrosis is not necessary for microsphere loss. Murdock and Cobb (79) reported a maximum 9 μm microsphere loss of 22% from ischemic myocardium in the dog. Though this loss was statistically significant, the absolute difference in terms of blood flow ranged from 0.035 to 0.083 ml per minute per gram. They emphasized that this small loss should not interfere with the qualitative interpretation of serial blood flow measurements made with microspheres injected following infarction. Finally, Bishop (15) and Jugdutt et al. (60), and Reimer and Jennings (91) have indicated that in chronic infarcts with scar formation and tissue reabsorption, there may actually be an apparent microsphere gain relative to noninfarcted areas.

Investigators in our laboratory have been using radioactive microspheres to study regional blood flow. These microspheres are a useful experimental tool in

work designed to study the occurrence of functional obstruction to regional blood flow. Microspheres will be used to determine regional myocardial blood flow in canine myocardium subjected to ischemia and reflow. Whether or not reflow inhibition, no-reflow, occurs in this model will be determined. This information will then be related to ultrastructural changes occurring in the myocardium during no-reflow and the cause of this phenomenon will be determined.

CHAPTER II

REGIONAL MYOCARDIAL BLOOD FLOW FOLLOWING TEMPORARY ISCHEMIA

Introduction

Regional myocardial blood flow in both normal and ischemic myocardium is an area of great interest to numerous cardiovascular researchers. The recent introduction of coronary bypass surgery as a practical treatment in some cases of coronary heart disease and the introduction of streptokinase and other fibrinolytic agents have served to heighten the interest of these investigators in regional myocardial blood flow following ischemia. Several investigators (43,74,92) have reported that reperfusion following brief periods of ischemia reduces the amount of necrosis, and, therefore, the size of the infarct associated with this flow deficit. Others (19,52) have reported that reperfusion following short term ischemia increases the size of the infarct and makes it hemorrhagic. Still other investigators (30,64,68,74,101,131) have reported that the restoration of blood flow to myocardium made ischemic for a "critical" time period may not be possible or at least is very difficult. This phenomenon has been reported to occur in the kidney, skin, brain and adrenal gland of various animals and is called the "no-reflow" phenomenon (3,27,58,64,108,120).

Krug, de Roachemont and Korb (68) first suggested the no-reflow phenomenon could occur in the heart. They reported an inhibition of reperfusion in the subendocardium of cats following temporary ischemia lasting 60-120 minutes. Using acridine orange as an indicator, they found that 6 of 12 cats

studied 60-360 minutes after 60 minutes of occlusion had a continued deprivation of flow to the subendocardium. Following 120 minutes of temporary ischemia in 8 cats, all showed a continued subendocardial reflow defect for up to 360 minutes after release of the occlusion. Kloner, Ganote and Jennings (64) were the first to report that a similar phenomenon occurred in the dog myocardium. They used thioflavin-S and carbon black to assess the occurrence of reflow 5 and 20 minutes following 40 and 90 minutes of temporary ischemia. After 40 minutes of ischemia reflow occurred throughout the region at risk during the occlusion. However, following 90 minutes of ischemia the inner half of the myocardium at risk was not uniformly penetrated by their tracers after either 5 or 20 minutes of attempted reflow.

Neither of these studies provides an opportunity to quantitate the amount of ischemia necessary to produce the no-reflow phenomenon. Therefore, the relationship between the degree of ischemia and the time it must exist to produce this phenomenon is not known. Darsee and Kloner (30) have used microspheres to study this phenomenon in the canine myocardium. However, they did not relate the degree of ischemia to time with respect to the generation of the no-reflow phenomenon. The microsphere technique could serve as a useful tool in this type of study. The present study was designed to study the effect of temporary ischemia on regional myocardial blood flow and to quantitate the degree of ischemia and its duration necessary for the delaying or prevention of reperfusion. A second objective was to determine if reperfusion was inhibited following 30 minutes of myocardial ischemia in the dog as Krug et al. (66) has reported in the cat. Finally, in this experiment the relationship of inhibited reflow to endothelial integrity is examined.

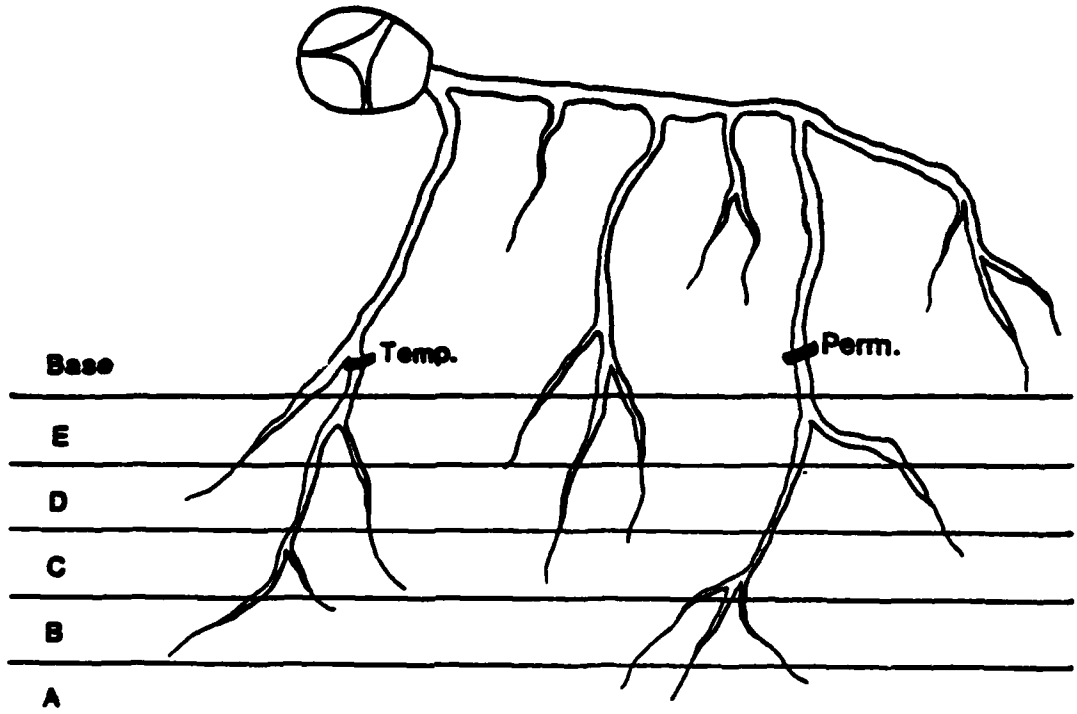
Methods

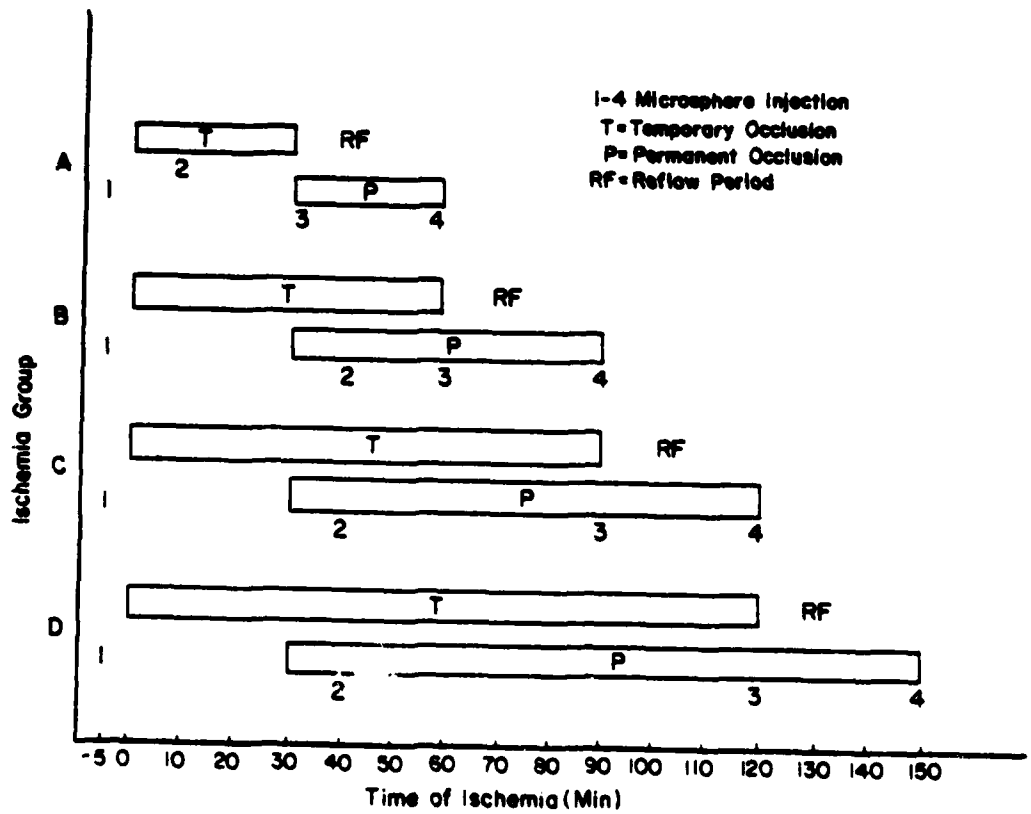
Experimental preparations: Forty healthy dogs, determined to be heartworm free, weighing 20-30 kg and of either sex were used. These animals were fasted overnight and then sedated with 20 mg droperidol and 0.4 mg fetanyl (1 cc Innovar-vet). Anesthesia was induced and maintained with halothane, nitrous oxide and oxygen using positive pressure respiration created with a Harvard respirator. This anesthetic regimen resulted in heart rates between 85 and 100 beats per minute and peak systolic pressure of 90-120 minutes. The chest was opened through the left fourth intercostal space and the heart was placed in a pericardial cradle. A diagonal branch of the left anterior descending coronary artery and a marginal branch of the left circumflex coronary artery with an intervening vessel were dissected proximally and a loose umbilical tape tie placed around them (Figure 1). A polyethylene catheter was secured in the left atrial appendage with a purse string suture for the injection of radioactive microspheres. Another catheter was placed in the terminal aorta through the right femoral artery for reference blood withdrawal. A catheter was also placed in the right femoral vein for the administration of 0.9% saline solution containing 1 mg/ml lidocaine to replace the amount of fluid lost due to hemorrhage and reference blood withdrawal. Left ventricular pressure was monitored using a Millar Mikro-Tip catheter pressure transducer model PC 350 and a Hewlett Packard 702A recorder. Lead II electrocardiograms were recorded on a Burdick EK 4 electrocardiograph. Lidocaine, 1 mg/Kg intravenously, was administered when needed to control arrhythmias.

Experimental design: Dogs were randomly assigned to four experimental groups: Group A = 30 minutes of ischemia, Group B = 60 minutes of ischemia, Group C = 90 minutes of ischemia, and a second Group A = 120 minutes of ischemia (Figure 2).

Figure 1 - Vessel Occlusion. Ligation of a diagonal branch of the left anterior descending coronary artery (temporarily) and a marginal branch of the left circumflex coronary artery (permanently). After the experiment, hearts were sliced into 5 mm sections (A-E).

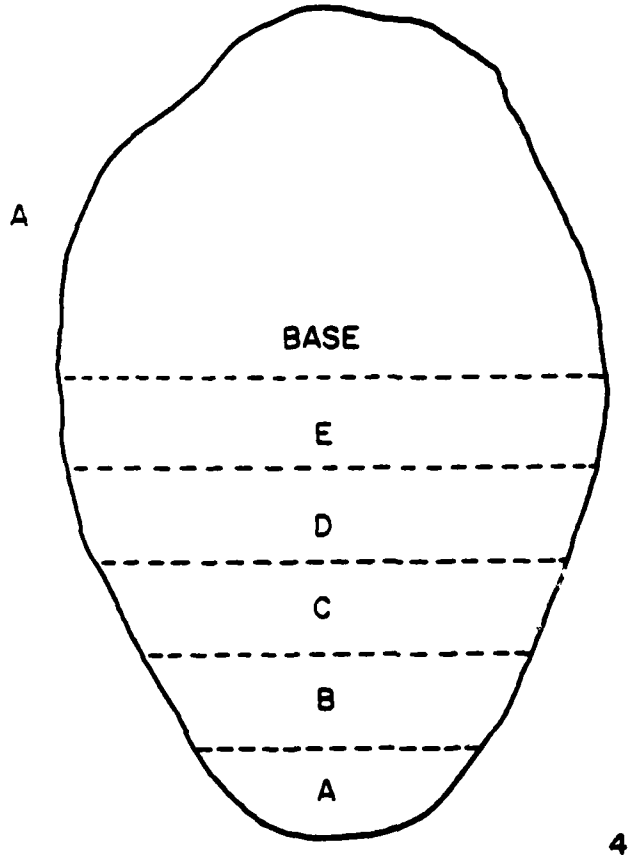
Figure 2 - Experimental Design. Ischemia Groups A-D represent 30,60,90,120 minutes of ischemia, respectively.


Base
Temp.
Perm.
E
D
C
B
A

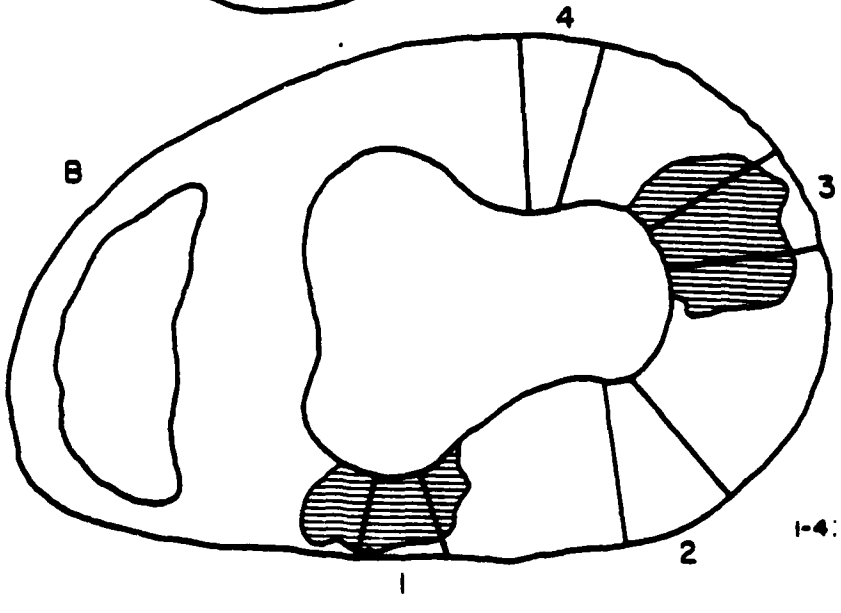

1-4 Microsphere Injection
T=Temporary Occlusion
P=Permanent Occlusion
RF=Reflow Period
Ischemia Group
A
B
C
D
T
RF
P
1
2
3
4
-5 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
Time of Ischemia (Min)

Regional blood flow and endothelial integrity: Following control electrocardiographic and left ventricular pressure recordings, radioactive microspheres were injected into the left atrium to determine preocclusion control regional myocardial blood flow. Microspheres were also given to group A animals ten minutes following the occlusion of the temporarily occluded vessel at the release of the temporary occlusion (beginning of the permanent occlusion) and at the end of the experiment. Groups B, C, and D received microsphere injections 40 minutes following ligation of the temporarily occluded vessel (10 minutes after ligation of the permanently occluded vessel), at the release of the temporary occlusion and at the end of the experiment. Standard microsphere techniques were used (15,20,31,50). Approximately 8×10^6 polystyrene nonbiodegradable 9 ± 1 μm tracer microspheres (3M) were injected. Microspheres were labelled with either cerium-141(Ce-141), chromium-51 (Cr-51), strontium-85 (Sr-85) or scandium-46 (Sc-46). They were suspended in 10% dextran and 0.05% Tween-80 surfactant to prevent aggregation. The order of microsphere injection was random. Prior to injection they were agitated on a vortex (Model S8223, Scientific Products) to further minimize clumping or settling. Absence of clumping was determined microscopically. Microspheres were injected from a specially constructed injector vial with 15 ml of isotonic saline into the left atrium. Five seconds before each injection, withdrawal of the reference blood samples was began. These samples were withdrawn at a rate of 7-10 ml per minute into three test tubes for one minute each using a polystaltic pump (Buchler Instruments). To study endothelial integrity a solution of 2% Evans blue biological stain (Fischer Scientific), 1 ml/Kg, was injected intravenously five minutes prior to the termination of the experiment during the reflow period. This invariably caused the heart to become blue, but its normal color returned before the end of the experiment.

Figure 3 - Heart Slices and Electron Microscopic Sampling. A. Hearts were sliced from apex to bane in 5 mm transverse sections. B. One milimeter thick transmural samples for electron microscopy (EM) studies were taken from ischemic and nonischemic areas.



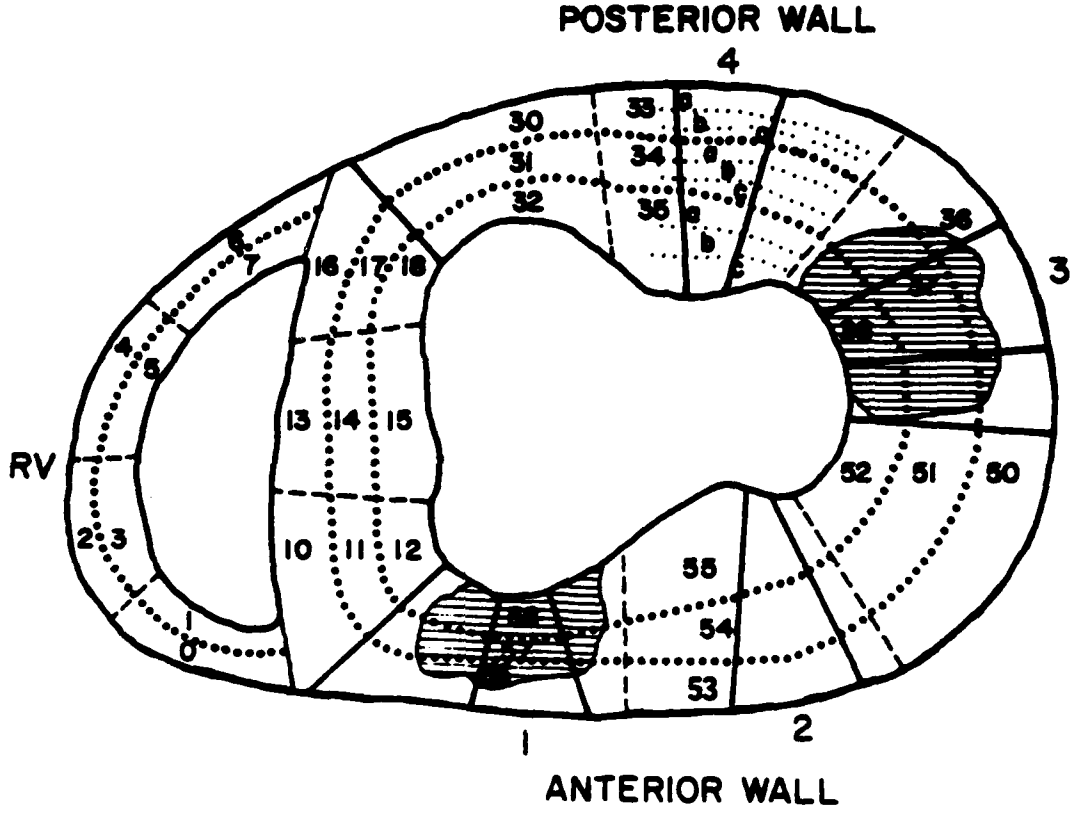
A-E: Heart Slice Identification



1-4: EM Samples

Hatching indicates ischemic area

Figure 4 - Sampling for Microsphere Counting. Slices containing ischemic tissue were sectioned into numerous samples circumferentially and then each of these was subdivided into subepicardial, midwall and subendocardial sections. 1-4 around left ventricle indicates areas where EM samples were taken.



Hatching indicates ischemic area

After the final injection of microspheres hearts were stopped with KCl. The hearts were perfusion fixed in situ at 100-110 mm Hg through the ascending aorta with a 2% solution of phosphate buffered paraformaldehyde and glutaraldehyde 338 mOsm after first being perfused with physiological saline. The fixed hearts were rapidly removed from the chest, blotted dry, weighed and sliced from apex to base in transverse 5 mm sections (Figure 3A). Horizontal 1 mm thick transmural sections for transmission electron microscopy (TEM) were taken from the center of temporary and permanent ischemia zones and from normal areas from several slices (Figure 3B).

The location of Evans blue dye was recorded on slice tracings. Slices containing portions of either ischemia zone were then sectioned into numerous samples around the wall (Figure 4). Each of these samples was further divided into subepicardial, midwall and subendocardial sections. A uniform numbering system was used as indicated in figure 4. Eight samples randomly chosen were weighed and dried along with all other samples. All dried tissue samples were then weighed and the difference between dry weight and wet weight for the eight randomly chosen samples was used to calculate the amount of water present in all samples. This information was then used in our computer blood flow program to convert tissue dry weight to wet weight. The radioactivity in all tissue samples and reference blood samples was determined with a Picker Autowell gamma counter and a model 401D Technical Measurement Corporation (Northhaven, Conn.) Pulse Height Analyzer. Counts in specific channel groups were summed with a PDP 8I computer, stored in a Texas Instruments Silent 700 bubble memory and transmitted via phone line to a Honeywell 560 computer for analysis. Blood flow for each sample was calculated according to methods described by Rudolph and Heymann (98) using the reference organ technique. Data were further analyzed within individual animals using specific programs written for use with

the Honeywell 560. Data were analyzed within and between groups using Student's nonpaired ttests, one way and two way analysis of variance where appropriate.

Infarct size determination: The percentage of the left ventricle made ischemic was calculated for both temporarily and permanently ischemic areas using the during the control pre-ischemic period. In order to compensate for possible alterations in blood flow due to experimental procedures, ischemic tissue flow was also determined as a percentage of flow in non-ischemic tissue at the same time period. In order to compensate for possible alterations in blood flow due to experimental procedures, ischemic tissue flow was also determined as a percentage of flow in non-ischemic tissue at the same time period.

Results

Experimental groups: Of the 40 dogs used 8 were excluded because of ventricular fibrillation. Fibrillation occurred in the first 5 minutes after the occlusion of one or both vessels or a few minutes after release of the temporary occlusion. Six dogs were excluded because the area of ischemia was smaller than our sampling technique could measure or was non-existent. Two dogs were excluded because they had multifocal myocardial scars associated with several shotgun pellets. The remaining 24 dogs were randomly assigned to Groups A-D. Several dogs developed ventricular tachycardia a few minutes following release of their temporary occlusion. This arrhythmia in all cases was controlled with Lidocaine. One dog from Group C was later excluded after it was determined that it inadvertently had not been given one microsphere injection.

Infarct sizes: There was no significant difference between the mean size of the temporarily and permanently ischemic zones within each group using Student's t-test and a p value $\leq$ 0.05. (Table 1) Maximum infarct size was determined as the sum of total ischemic tissue identified using original tissue blood flow or mean IVS blood flow as control. The identification of ischemic tissue did not always

Table 1. Infarct Size

	Maximum Infarct Size +	Temporary Ischemia % Control Flow	Temporary Ischemia % Mean IVS Flow *	Permanent Ischemia % Control Flow	Permanent Ischemia % Mean IVS Flow
30 minutes (Group A)	21.8 ± 5.56	7.0 ± 4.76	7.0 ± 2.45	9.5 ± 5.45	9.8 ± 4.86
60 minutes (Group B)	14.0 ± 6.63	5.8 ± 3.03	5.8 ± 1.30	4.0 ± 4.12	3.9 ± 2.12
90 minutes (Group C)	20.3 ± 6.40	10.0 ± 5.48	8.5 ± 6.24	6.8 ± 2.06	5.0 ± 1.14
120 minutes (Group D)	15.0 ± 8.19	8.5 ± 7.58	8.0 ± 7.29	5.0 ± 2.61	3.0 ± .63

Values are mean ± S.D.
+ % of left ventricle
* IVS = Intraventricular septum

coincide when these two methods were used. Therefore, the maximum infarct size in all four groups is larger than the sum of the temporarily and permanently ischemic areas. The mean maximum infarct size was less than 22% of the total left ventricular mass in all four groups.

Endothelial integrity and ischemia: The distribution of grossly visible Evans blue stain within the groups is shown in figure 5. None of the areas with permanent ischemia had visible dye. Temporarily ischemic samples from all four groups were subdivided based on the degree of ischemia into tissues receiving $\leq$25%, >25$\leq$50% and >50% of their individual control blood flow. There was no difference in the degree of ischemia suffered by these samples when either their original control tissue flow or mean IVS flow was used as control flow. Only data generated using original tissue blood flow for control flow are presented. None of 63 temporarily ischemic tissues from Group A animals had dye. Two subendocardial samples (blood flow $\leq$ 25% of control) were stained from a total of 57 samples from Group B animals. Four of 48 Group C temporarily ischemic samples contained stain. Evans blue dye was present primarily in subendocardial and corresponding midwall samples. Seventeen of 51 Group D samples contained stain. Loss of endothelial integrity, i.e., blueing, was directly related to the length of the ischemic period, and was nearly always limited to tissues with $\leq$ 25% normal blood flow.

Regional blood flow: Figures 6-9 are representative of the blood flow pattern observed in all Group A samples. Numbers used to identify tissue samples correspond to those used in figure 4. However, there are more samples represented in figures 6-9 because this slice was sectioned into smaller more numerous samples.

Control blood flow was quite uniform transmurally in most dogs from all four groups (Figure 6). There was, however, some variation with occasionally a higher flow in the subepicardium than that present in the subendocardium. This

Figure 5 - Vascular leakage during reflow of ischemic tissue shown as a percentage of control flow. Each column represents a different level of ischemia. The number of samples with blueing/total number of samples is shown above each column.

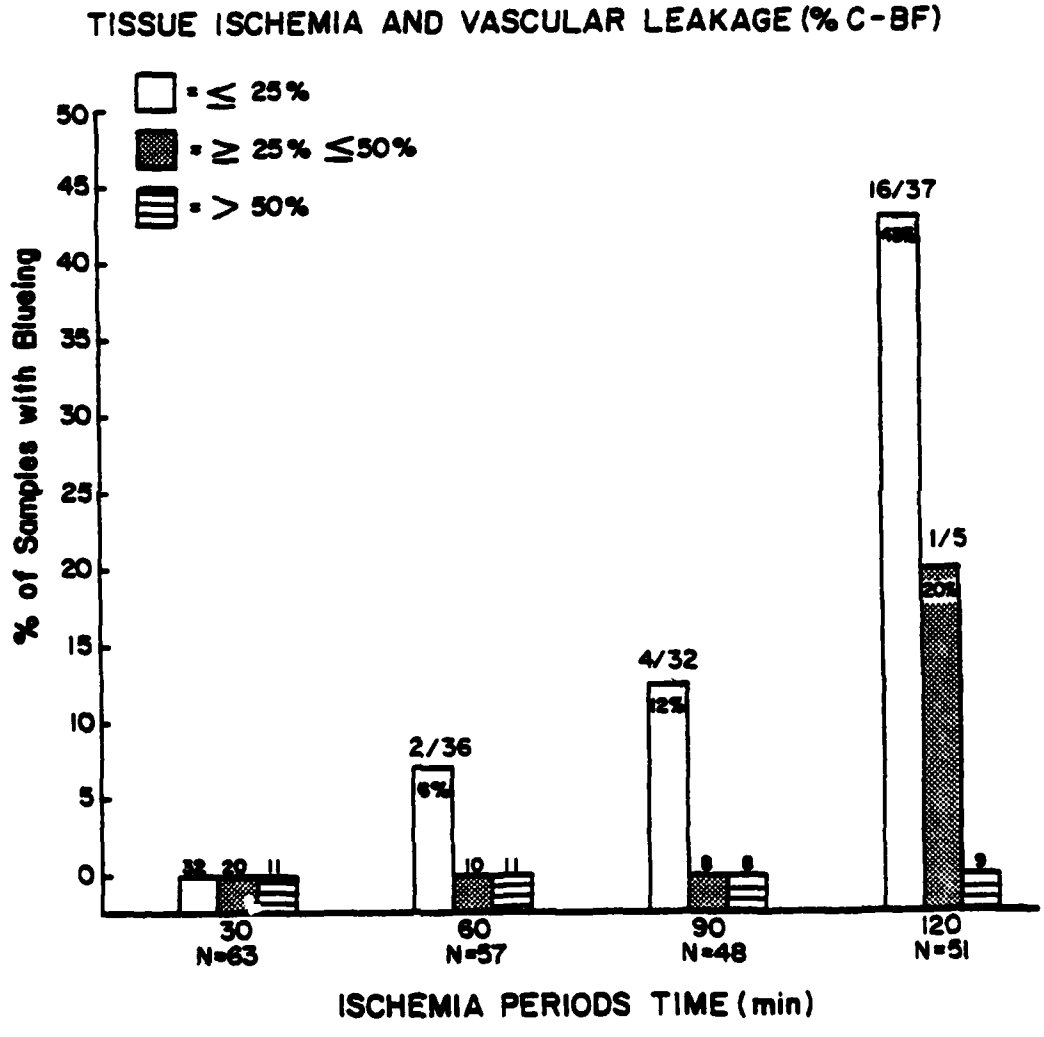
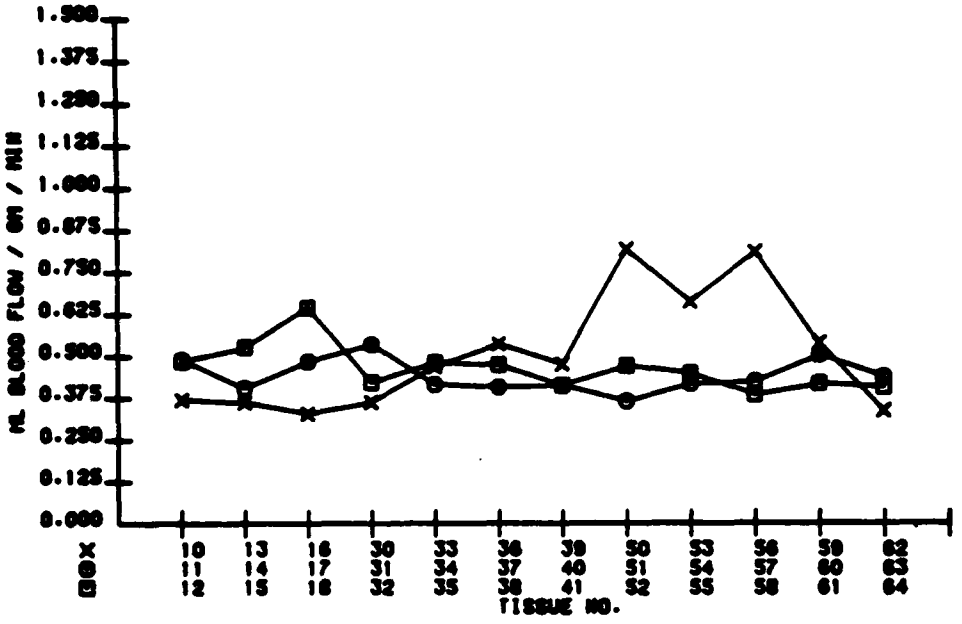

TISSUE ISCHEMIA AND VASCULAR LEAKAGE (% C-BF)
□ = ≤ 25%
▩ = ≥ 25% ≤50%
▤ = > 50%
% of Samples with Blueing
50
45
40
35
30
25
20
15
10
5
0
16/37
1/5
4/32
2/36
30
N=63
60
N=57
90
N=48
120
N=51
ISCHEMIA PERIODS TIME (min)

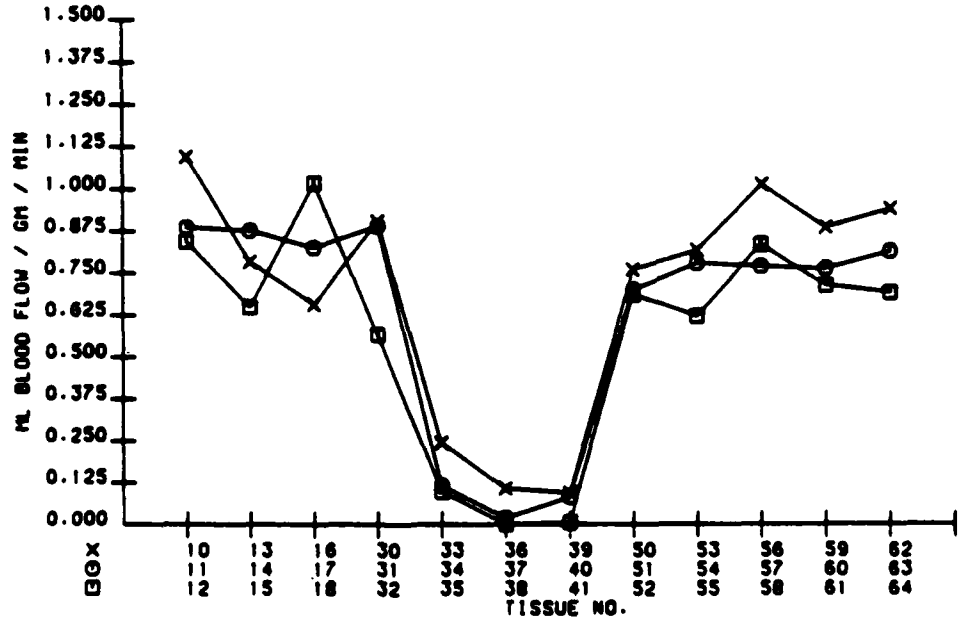
Figure 6 - First (control) Microsphere Injection. Control blood flow in an animal from Group A. X, subepicardium; midwall, and subepicardium. Numbers 10-18 corresponds to intraventricular septum samples; 30-41 posterior wall and posterior-lateral wall junction and 50-64 tissues from anterior-lateral wall junction and the anterior wall.

Figure 7 - Second Microsphere Injection. Samples 33-41 are supplied by the marginal branch of the LCCA which is occluded at this time. These tissues are severely ischemic.

SLICE D 221 - 399 DOG D6128



SLICE D 150 - 220 DOG D6128



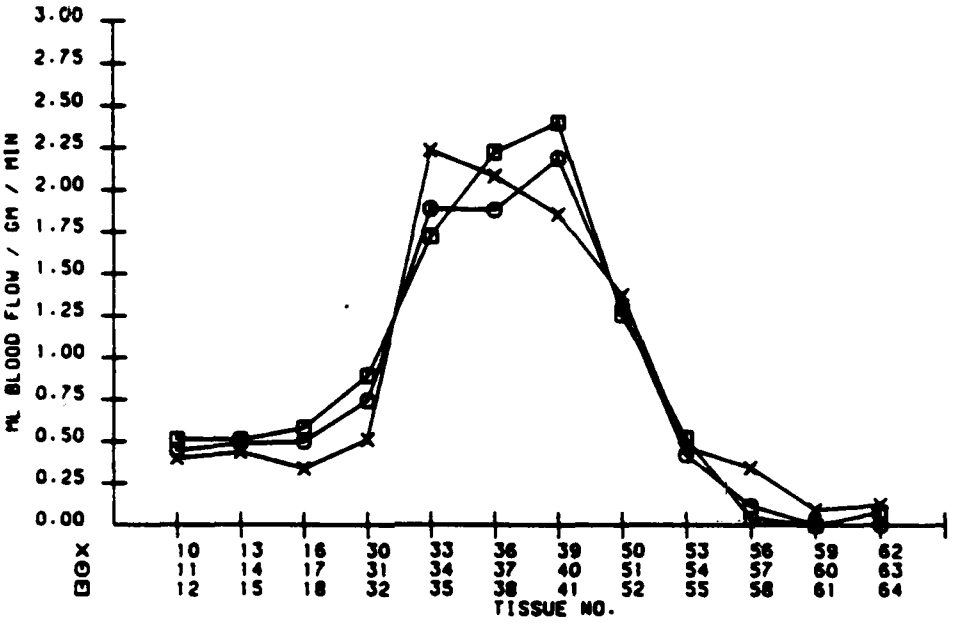
finding was often focal and associated with trauma to the epicardium resulting from vessel isolation (Figure 6). Most dogs demonstrated a subendocardial to subepicardial blood flow ratio close to unity. Severe transmural ischemia occurred with vessel ligation (Figure 7). This is well demonstrated in samples 33-41 during ligation of a marginal branch of the left circumflex coronary artery. Portions of the posterior wall and its lateral wall junction have been made severely ischemic. Samples 36-38 are located in the center of the ischemia zone. Release of the temporary occlusion in all Group A dogs resulted in an immediate manyfold reactive hyperemia increase in flow above control level (Figure 8). At this same time permanent ligation of a diagonal branch of the anterior descending coronary artery produces transmural ischemia in tissues supplied by this vessel. Tissues 56-64 demonstrate this well and samples 59-61 are located in the center of this ischemia zone (Figure 8). Note also that the IVS in this slice is not made ischemic by ligation of either vessel (Figures 7 and 8). Thirty minutes after the release of the temporary occlusion, hyperemia had abated in nearly all previously ischemic samples (Figure 9). Those located at the periphery of the ischemic zones frequently manifested hyperemia. Sample 55, a subendocardial sample, provides good demonstration of this phenomenon (Figure 9).

A graphic presentation of the changes in blood flow occurring in samples 36-38 of slice D is presented in figure 10. Blood flow changes here are calculated as a percentage of the original control tissue flow. Ischemia is present during vessel ligation. Release of the ligature causes a reactive hyperemia in these samples. Thirty minutes later flow has returned to near normal control level. Figure 11 is a similar presentation of blood flow changes in three permanently ischemic tissues. Transmural ischemia like that present in temporarily and permanetly ischemic samples of Group A was also present in samples from Groups B-D. The temporarily and permanently occluded vessels were both occluded during the

Figure 8 - Third Microsphere Injection. Immediate reactive hyperemia seen in sample 33-41 with release of marginal branch of LCCA. Samples 57-64 are severely ischemic due to ligation of a diagonal branch of LAD.

Figure 9 - Fourth Microsphere Injection. Thirty minutes after release of the temporarily occluded marginal branch of the LCCA, but the diagonal branch of the LAD is still occluded. Subendocardium (55) bordering permanent ischemia area shows hyperemia.

SLICE D 92 -135 DOG D6128



SLICE D 35 -65 DOG D6128

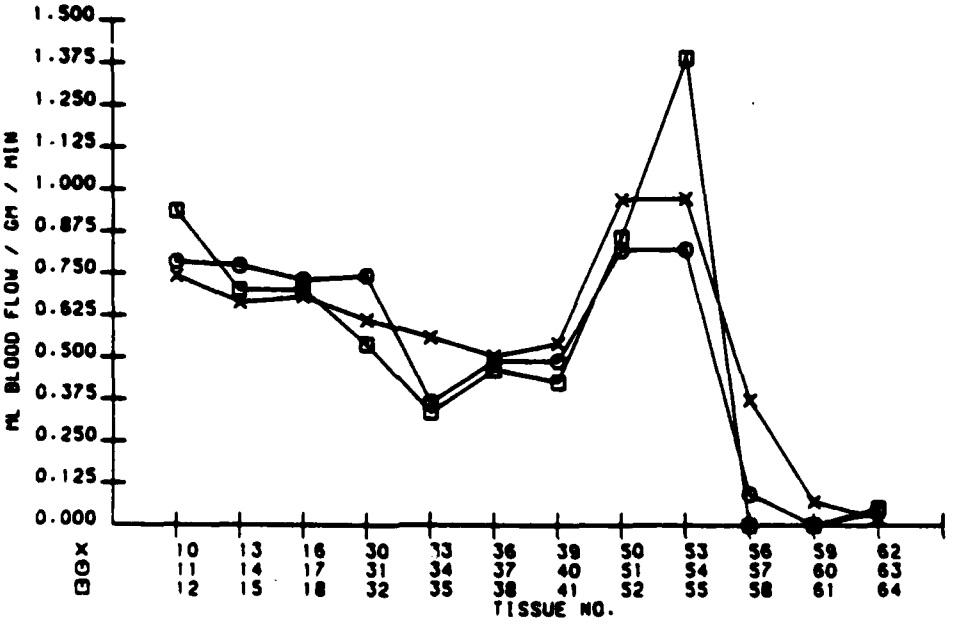


Figure 10 - Temporarily Ischemic Tissue Blood Flow Changes. Flow changes in tissues 36 (X, subepicardium); 37 (0, midwall), and 38 (■ , subendocardium) from dog D6128 Slice D.

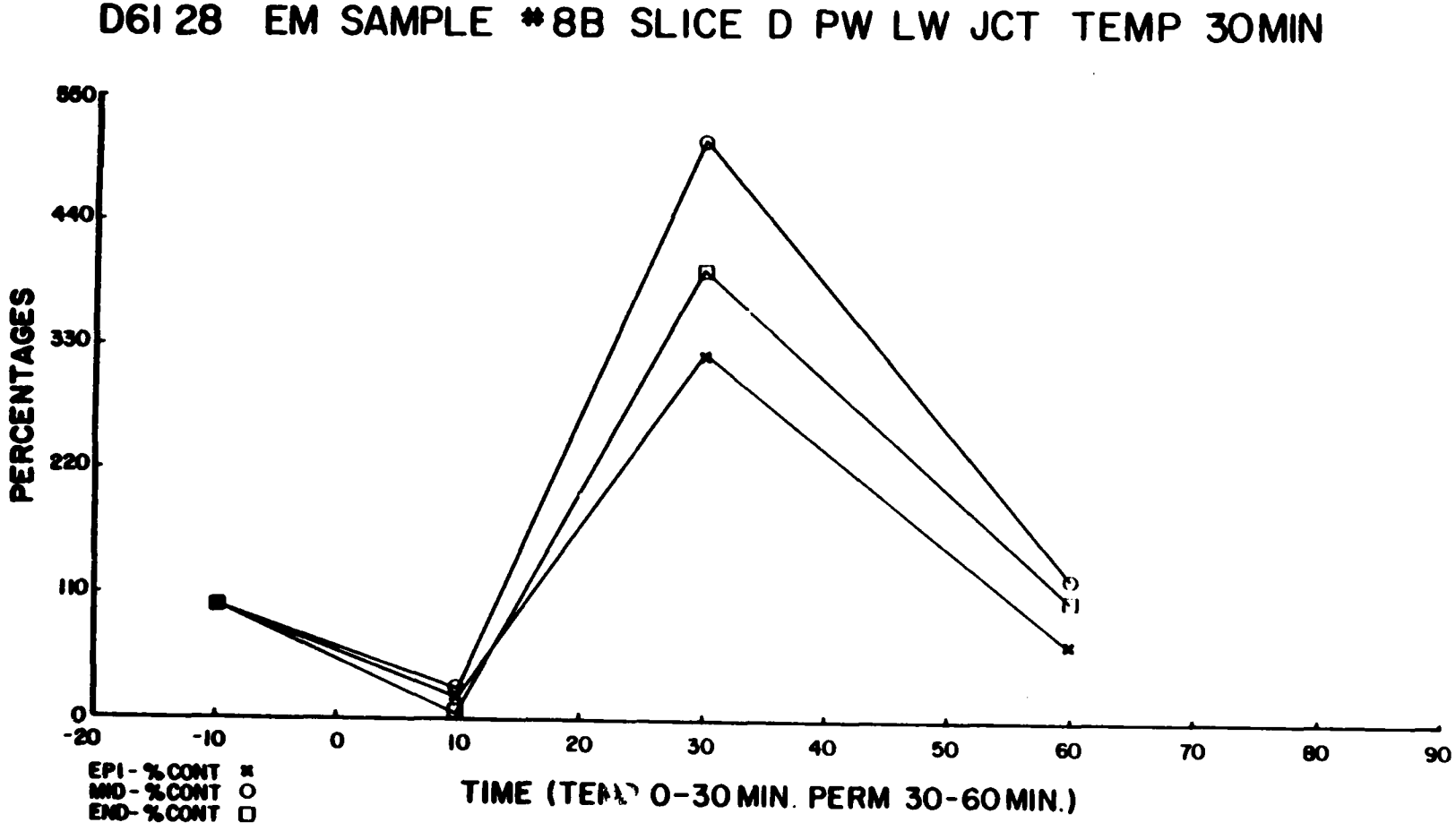
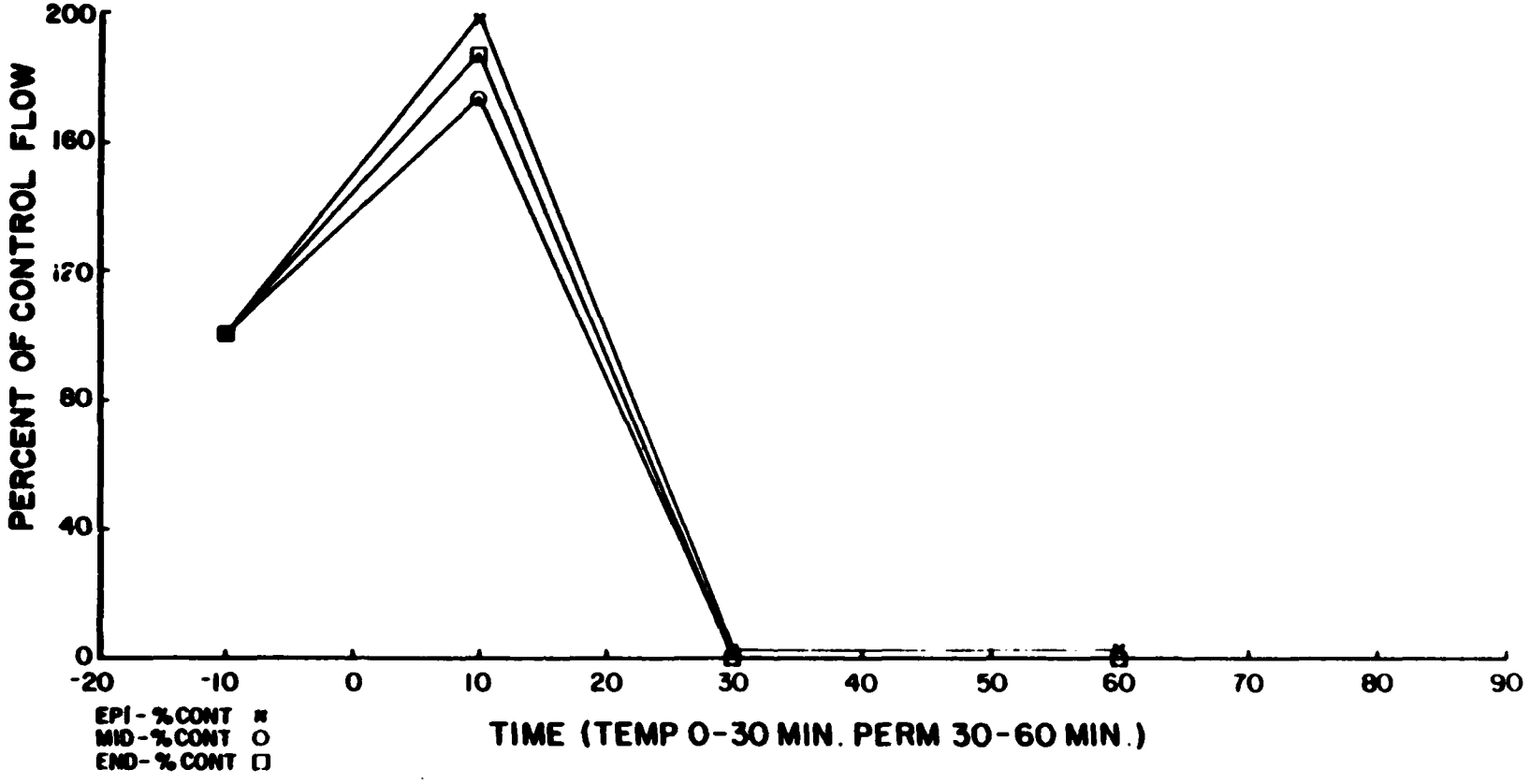

D6128 EM SAMPLE *8B SLICE D PW LW JCT TEMP 30MIN
PERCENTAGES
550
440
330
220
110
0
-20
-10
0
10
20
30
40
50
60
70
80
90
EPI - %CONT *
MID - %CONT O
END - %CONT □
TIME (TEMP 0-30 MIN. PERM 30-60 MIN.)

Figure 11 - Permanently Ischemic Tissue Blood Flow Changes. Flow changes in tissues 59 (X, subepicardium); 60 (0, midwall) and 61 (■, subendocardium) from dog D6128 Slice D.

D6128 EM SAMPLE #4 SLICE B AW PERM 30 MIN



second microsphere injection in these latter groups. Therefore, portions of the anterior and posterior wall were ischemic simultaneously in these groups (Figure 12).

Hyperemia response and ischemia: The relationship between the degree of ischemia and hyperemia was determined for Groups A-D using all temporarily ischemic samples (Table 2). All Group A samples had a marked reactive hyperemia response immediately following release of the temporary occlusion. The level of hyperemia response in these samples was inversely related to their degree of ischemia (Figure 13). Samples with > 50% control flow during vessel ligation had the greatest hyperemia response.

The samples from Group B behaved in a strikingly different manner (Figure 14). Samples receiving > 50% of control blood flow during their ischemia episode had a drastic change in their hyperemia response. Only 54% of these samples responded with a reactive hyperemia flow > 150% of control flow. Forty-five percent of these samples had a blunted hyperemia response ($\leq$ 150%). Nine percent of these samples actually received $\leq$ 60% of their control flow immediately following the end of vessel occlusion. Tissues receiving > 25 $\leq$ 50% of their control flow during the period of ischemia had an even more blunted hyperemia response. Thirty percent of the samples with ischemia at the middle level had a hyperemia response $\leq$ 60% of control flow. This is slightly more than a three-fold increase over the percentage with this response after ischemia of > 50% control blood flow. The most severely ischemic tissue in Group B responded similarly to tissues in the middle level ischemia group.

The most striking development in Group C samples was a mild increase in the percentage of tissue with reflow > 300% of control flow (Figure 15). This change was more prominent in the most severely ischemic tissues. There was a corresponding decrease in the percentage of samples (22%) having a hyperemia

Figure 12 - Second Microsphere Injection in a Group B Dog. A diagonal branch of the LAD and a marginal branch of the LCCA are occluded. X, subepicardium; 0, midwall and ■, subendocardium.

SLICE C 92 -135 DOGD5909

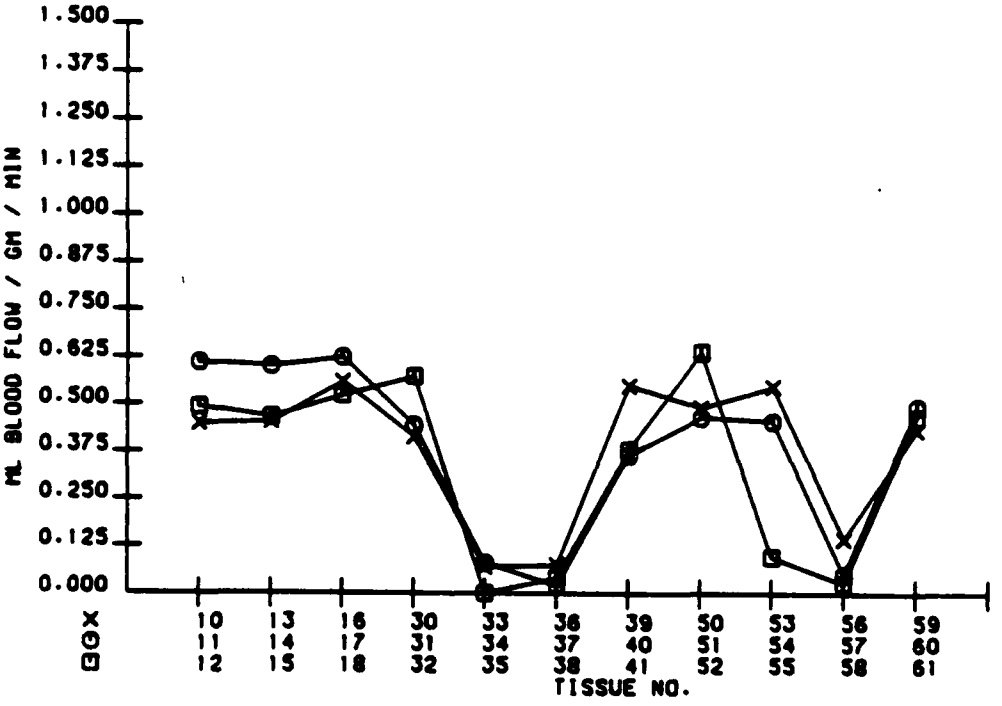


Table 2. Reactive Hyperemia and Ischemia

	Degree of Ischemia	Level of Hyperemic Response			
		≤60%*	>60%≤150%	>150%≤300%	>300%
	≤25%*	——	——	9/32(28%)**	23/32(72%)
30 min.	>25%≤50%	——	——	5/20(25%)	15/20(75%)
Ischemia	>50%	——	——	——	11/11(100%)
	≤25%	11/36(31%)	5/36(14%)	9/36(25%)	11/36(31%)
60 min.	>25%≤50%	3/10(30%)	1/10(10%)	3/10(30%)	3/10(30%)
Ischemia	>50%	1/11(9%)	4/11(36%)	3/11(27%)	3/11(27%)
	≤25%	7/32(22%)	1/32(3%)	5/32(16%)	19/32(59%)
90 min.	>25%≤50%	2/8(25%)	3/8(38%)	——	3/8(38%)
Ischemia	>50%	——	2/8(25%)	3/8(38%)	3/8(38%)
	≤25%	7/37(19%)	2/37(8%)	——	27/37(73%)
120 min.	>25%≤50%	3/5(60%)	——	——	2/5(40%)
Ischemia	>50%	——	——	1/9(11%)	8/9(89%)

* Percent of original tissue flow

** Numerator = No. with this amount hyperemia
Denominator = No. with this degree of ischemia
No. in parenthesis = % numerator is of denominator

Figure 13 - Reactive Hyperemia and 30 Minute Ischemia. Four columns represent varying levels of immediate reactive hyperemia in tissue suffering one of three levels of ischemia. Ischemia and hyperemia blood flow shown as a percent of control flow. All ischemic samples demonstrate a moderate to excellent hyperemia response.

REACTIVE HYPEREMIA AND 30min ISCHEMIA (% C-BF)

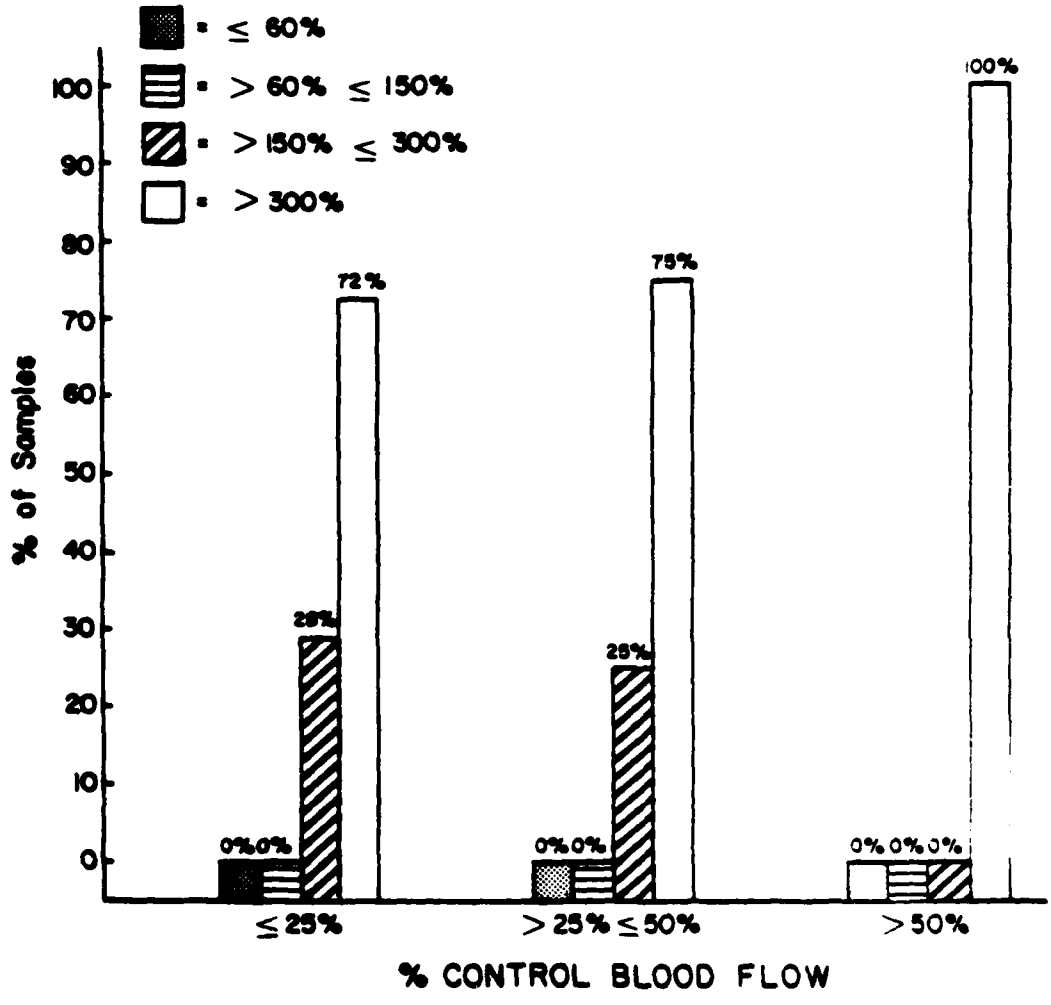


Figure 14 - Reactive Hyperemia and 60 Minute Ischemia. Blunting of the hyperemia response seen in samples from all ischemia levels.

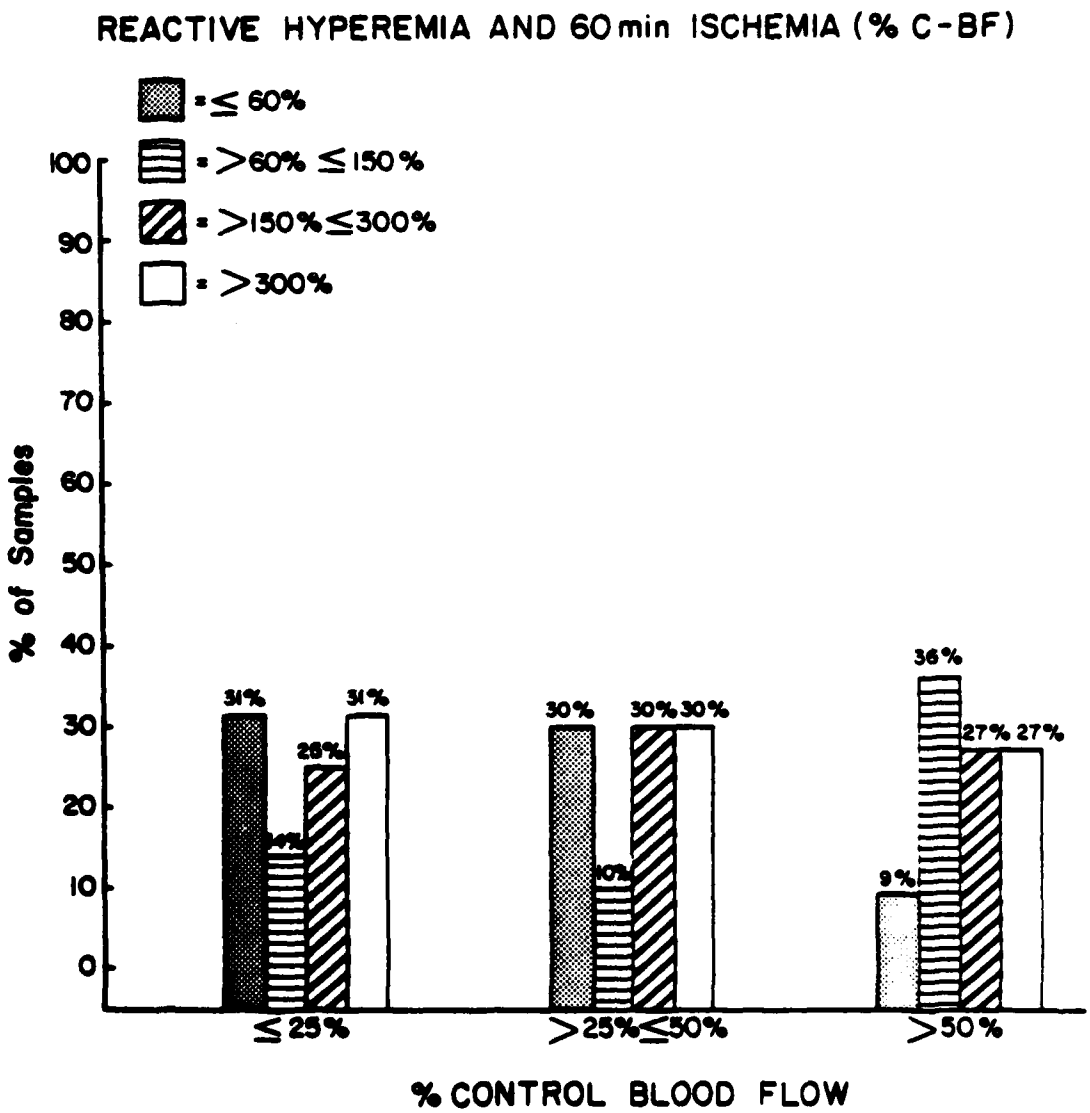
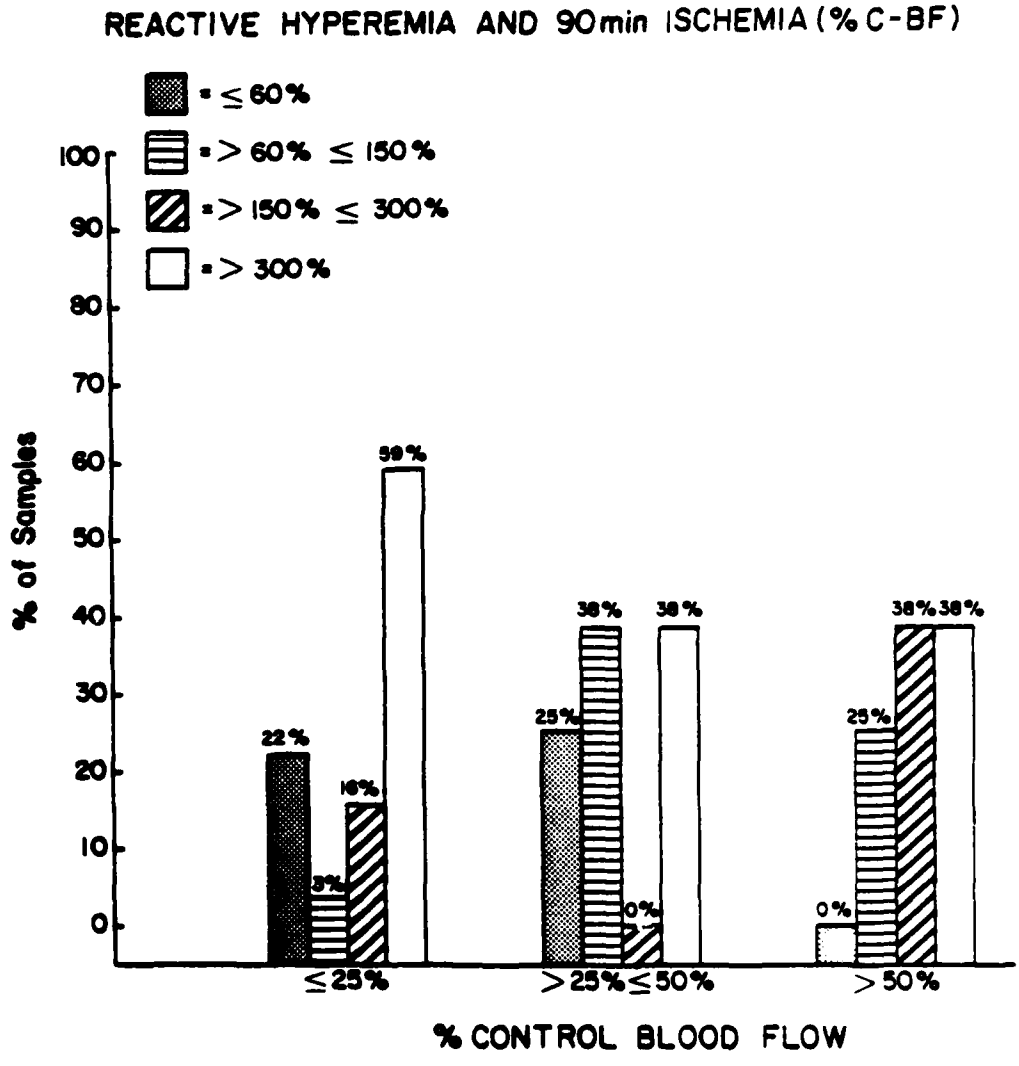

REACTIVE HYPEREMIA AND 60 min ISCHEMIA (% C-BF)
= ≤ 60%
= >60% ≤150%
= >150%≤300%
= >300%
% of Samples
100
90
80
70
60
50
40
30
20
10
0
31%
14%
28%
31%
30%
10%
30%
30%
9%
36%
27%
27%
≤25%
>25%≤50%
>50%
% CONTROL BLOOD FLOW

Figure 15 - Reactive Hyperemia and 90 Minute Ischemia. Increase in reactive hyperemia response, especially in severely ischemic samples.


REACTIVE HYPEREMIA AND 90min ISCHEMIA (% C-BF)
= ≤ 60%
= > 60% ≤ 150%
= > 150% ≤ 300%
= > 300%
% of Samples
100
90
80
70
60
50
40
30
20
10
0
22%
3%
16%
59%
25%
38%
38%
0%
0%
25%
38%
38%
≤ 25%
> 25% ≤ 50%
> 50%
% CONTROL BLOOD FLOW

response of $\leq$ 60% of control flow. These trends were even more pronounced in samples from Group D (Figure 16). Eighty-nine percent of tissues receiving > 50% of control blood flow during ischemia had an initial hyperemia flow > 300% of control flow. The most severely ischemic tissues in this group now had a distinctly better hyperemia response. Though 56% of the most severely ischemic tissues of Group B had a hyperemia response > 150% of control flow, this value was 75% for Group C and 73% for Group D samples (Figures 14,15,16). The inhibition of reflow was transient in all temporary samples but one. In this lone Group C sample reflow was still inhibited 30 minutes following release of the occlusion. There was no blue stain present in this sample.

Subendocardial and subepicardial hyperemia response: Subtracting the degree of ischemia (percent of control blood flow) present in the subendocardium from that present in the subepicardium provides a picture of the transmural nature of the ischemia occurring across the wall. Eighty percent of the subendocardial samples were either more ischemic than their subepicardial counterparts or the degree of ischemia was uniform throughout. When temporary occlusion ended, 82% of the subepicardial samples had a greater hyperemia response than did corresponding subendocardial samples. This relationship was present even in samples with inhibited reflow. However, thirty minutes after release of the temporary occlusion, blood flow was greater in the subendocardium than in the subepicardium in over 85% of our samples. Subendocardial samples from Groups C and D with an initially blunted hyperemia response frequently demonstrated moderate levels of hyperemia at this time (Figure 17). Usually the subepicardial blood flow returned to near control level..

Discussion

The reactive hyperemia response in all tissues ischemic for 30 minutes, at all levels of ischemia, like the endothelial integrity of vessels suppling them was

Figure 16 - Reactive hyperemia and 120 Minute Ischemia. Prominent increase in reative hyperemia response in severely ischemic and mildly ischemic samples. Increased severe blunting of this response in moderately ischemic samples.

REACTIVE HYPEREMIA AND 120 min ISCHEMIA (% C-BF)

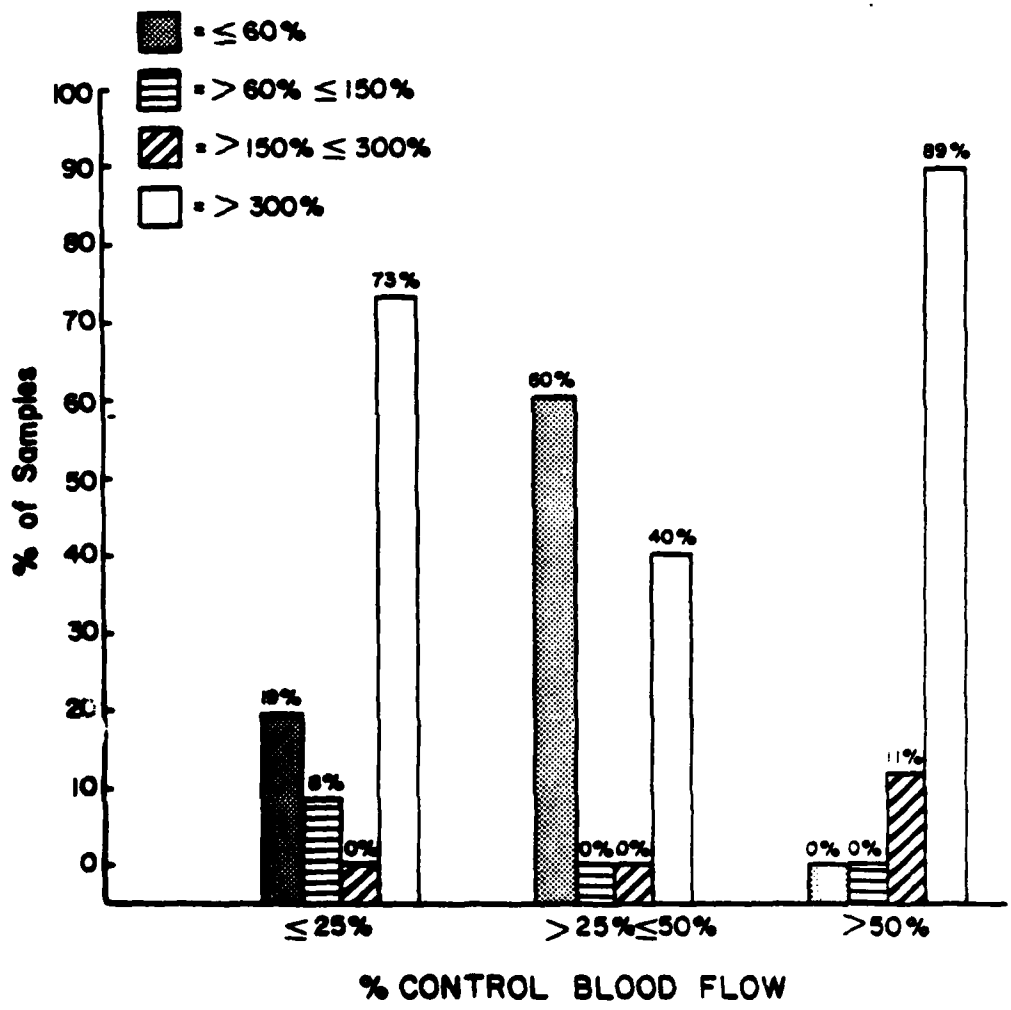
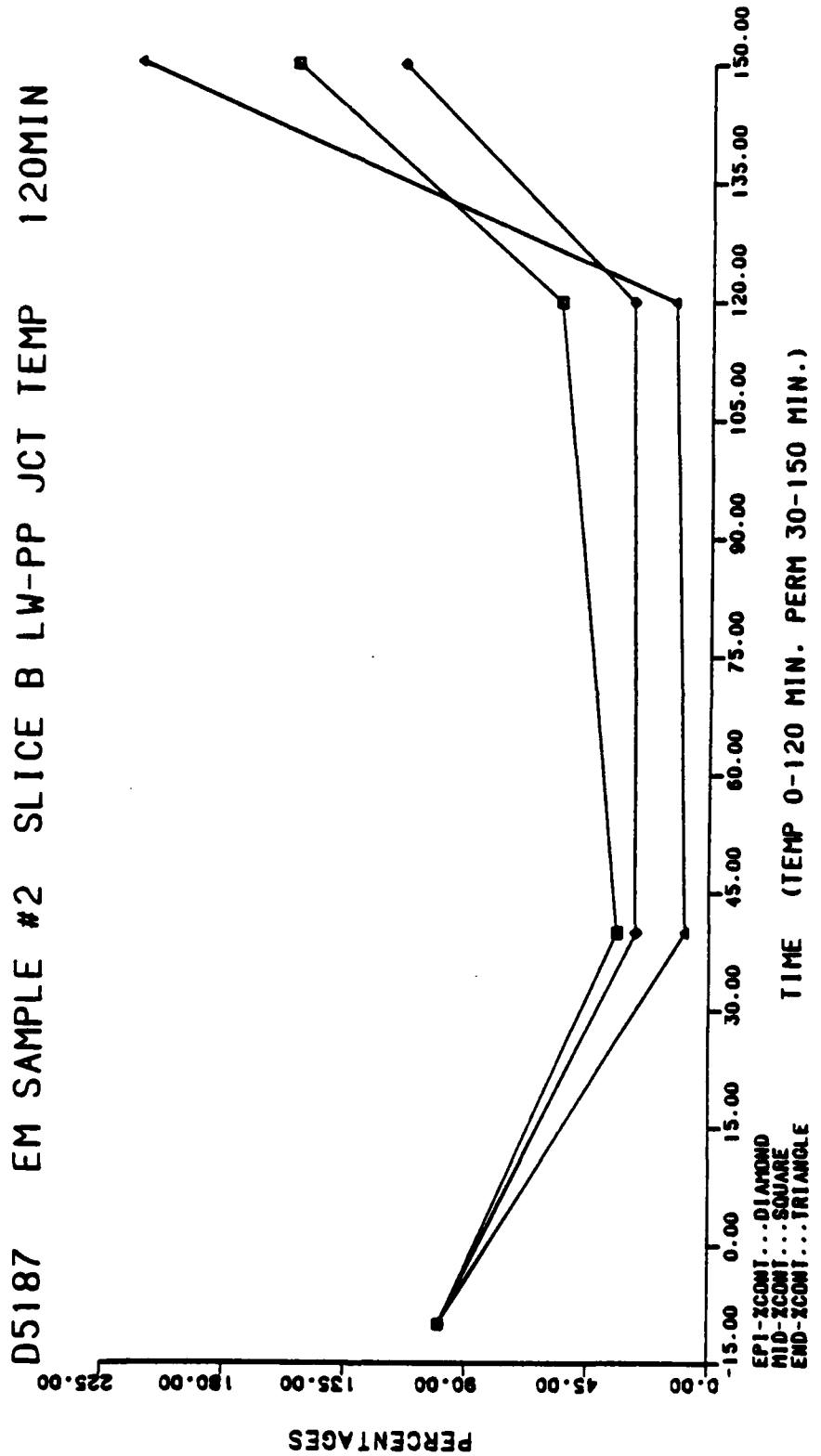


Figure 17 - Transient Inhibition of Reflow Following 120 Minutes of Ischemia. Blood flow pattern to lateral wall-posterior papillary junction (LW-PPJCT) after 120 minutes of temporary ischemia. Hyperemia of subendocardium after 30 minutes of reflow. Blood flow in the subepicardium frequently returned only to near normal levels.


D5187 EM SAMPLE #2 SLICE B LW-PP JCT TEMP 120MIN
PERCENTAGES
0.00
45.00
90.00
135.00
180.00
225.00
-15.00
0.00
15.00
30.00
45.00
60.00
75.00
90.00
105.00
120.00
135.00
150.00
TIME (TEMP 0-120 MIN. PERM 30-150 MIN.)
EPI-%CONT:..:DIAMOND
MID-%CONT:..:SQUARE
END-%CONT:..:TRIANGLE

normal. Jennings et al. (56) have reported that myocardial cells may be irreversibly damaged after 20-40 minutes of ischemia. It is apparent from the present study that capillaries and venules supplying these myocytes are still capable of controlling the escape of fluid and proteins extravascularly at this time. The pattern of reactive hyperemia seen following 60 minutes of occlusion was quite different from what was seen after 30 minutes. At one hour a transient severe blunting or inhibition of the hyperemia response had occurred in samples from all ischemia levels. In only one sample from this group was this inhibition still present 30 minutes after release of the occlusion. Samples from the 90 minutes ischemia group showed a small but definite trend toward a hyperemia response similar to the one seen following 30 minutes of ischemia. This was most apparent in the more severely ischemic samples.

The greater initial hyperemia response in tissues from Group C was not expected. In fact, a more pronounced decrease in the reactive hyperemia response was expected in these tissues, like that seen in tissues ischemic for one hour. Only tissues in the middle ischemic group had a more severe blunting of the hyperemic response. This trend of increased initial hyperemia following the termination of vessel occlusion was much more prominent in the 2 hour ischemia group. A much higher percentage of samples in this group of mildly ischemic tissue had a reactive hyperemia response >300% of control flow. In the most severely ischemic samples with 120 minutes of ischemia a hyperemia response >300% of control flow occurred much more frequently than after 60 or 90 minutes of ischemia. The inhibition of flow in these samples like that present in all samples, but one was transient in nature.

Olsson (81) has reported that the duration of ischemia and the responsiveness of coronary vessels to a dilator substance that accumulates during ischemia determines the degree of hyperemia response. In the present experiment the

problem is not the failure of vessels to dilate following ischemia. If this was true one would expect at least a return to control flow levels following release of the ligature. An increase in the amount of non-responsiveness as the duration of ischemia increased would also be expected. Instead the present experiment indicates that maximum inhibition of reflow following ischemia of $\leq$ 25% control blood flow occurs 60 minutes after vessel occlusion. This inhibition had already started to decrease during 90 minutes of ischemia. This experiment also demonstrates that peak inhibition of reflow occurs in tissues with $> 25 \leq 50\%$ of control flow 90-120 minutes after occlusion.

Bannerman, Powers and Cannon (5) recently reported that myocardial pH may be an important determinant of the hyperemia response following prolonged coronary occlusion. They reported that an inverse linear relationship existed between pH and coronary blood flow in postischemic myocardium. Our 30 minute ischemia group indicates that hyperemia is probably not controlled primarily by tissue pH. In this group of dogs hyperemia occurred in all samples. Furthermore, the highest percentage of samples with a reactive hyperemia response >300% of control flow was seen in the mildly ischemic tissues and not in the more severely ischemic ones. Though tissue pH was not measured in this experiment, it seems reasonable to assume that the greatest change in pH occurred in the most severely ischemic samples.

Olsson and Gregg (82) using an electromagnetic flowmeter confirmed that there is a phasic nature to the coronary reactive hyperemia response. In their experiments the amount of coronary flow occurring during the systolic portion of the coronary flow cycle reached peak flow levels during reactive hyperemia earlier than did its diastolic counterpart. Data from the present experiment clearly show immediately following the release of a temporary occlusion lasting 0.5 - 2 hours the subepicardium receives a greater proportion of coronary flow

than does the subendocardium. This occurs despite a greater degree of ischemia having occurred in the subendocardium. Bell and Fox (10) have shown that the subendocardium is perfused primarily during diastole. Maximum pressure at the inner surface of the myocardium may exceed systolic blood pressure and result in a virtual collapse of subendocardial intramural arteries (10). In the presence of increased vascular resistance in the subendocardium and peaking systolic flow the subepicardium receives a greater initial hyperemic blood flow than does the subendocardium. This relationship has reversed 30 minutes after release of the occlusion. It has been previously reported that the subepicardium has a greater hyperemic response in spite of greater subendocardium ischemia following vessel ligation (103). This has been attributed to the effects of extravascular resistances rather than to differences in the dilatory capacities of these two areas (105). As previously reported by Schaper (105) the hyperemic response in the subendocardium is longer lasting than the hyperemic response in the subepicardium in this experiment.

Riberio et al. (95) have reported that a hyperemic border zone occurs around ischemic myocardium and may persist for at least 45 minutes post-occlusion. This phenomenon was frequently seen in samples bordering ischemic tissue in this study. Whether or not this phenomenon was seen depended on the size of the border zone sample taken. If it was a very large sample then any focal hyperemic area present in it was masked by the normal flow reaching the rest of the sample.

Numerous investigators (21,30,64,68,101,109) have recently reported the occurrence of a no-reflow phenomenon occurring in the heart. Kloner et al. (64) summarized most of the suggested possible causes of this phenomenon. These include: 1) endothelial changes - swelling of endothelial cells and bleb formation might occlude capillaries, 2) changes in blood viscosity - red blood cell packing has

been seen in capillaries of the brain in areas demonstrating this phenomenon, 3) thrombosis - fibrin and platelet plugs that form due to stasis of flow might attach themselves to areas of vessel walls denuded of endothelium, 4) tissue edema - myocyte swelling following ischemia may occlude capillaries via subsarcolemmal bleb formation that might impinge upon capillaries from the outside. They conclude that there is no convincing evidence proving that any of these either alone or together are responsible for this phenomenon. Kloner et al. (64) do suggest however, that capillary damage is a major factor in the development of this phenomenon. They describe this phenomenon as occurring after 90 minutes of myocardial ischemia induced by ligation of the circumflex artery near its origin. Furthermore, they report this phenomenon is present 10-12 seconds, 5 minutes and 20 minutes after the initiation of reperfusion. In the present experiment a total inhibition of flow rarely occurred. Most frequently there was a slight elevation of blood flow above the level of collateral flow following release of the occlusion. It is quite possible that the use of dye markers, as in the work of Kloner et al. (64), may not have been sensitive enough to identify this flow.

The data presented indicate the present inhibition of flow is probably not due to some morphological aberration in capillary or venous structure. Capillary and venous function is closely tied to their morphology. The peak inhibition of reflow observed in these samples occurred earlier than did the peak increase in vascular permeability. These two were not always present in the same sample and are therefore probably unrelated. The transient nature of this reflow inhibition suggests that temporary vasoconstriction of precapillary vessels may be occurring. This vasoconstriction occurs most frequently following 60 minutes of severe ischemia and wanes during 90 and 120 minutes of ischemia. Less severely ischemic tissues are served by vessels that are maximumly constricted around 90 and 120 minutes after vessel occlusion.

Vasoconstriction has been reported to be a possible cause of the no-reflow phenomenon occurring in the brain (37,48). Ames et al. (3) were the first to demonstrate a no-reflow phenomenon in the brain of rabbits following five minutes of ischemia. Fischer et al. (37) studied rabbit brains following 30 minutes of global ischemia. They found that capillaries from areas demonstrating no-reflow were not occluded by blebs, endothelial swellings or luminal collapse. They proposed vascular constriction as an alternative cause of their observed "no-reflow." Hart et al. (48) found that vessels in the cerebral cortex of cats had thicker walls and smaller lumens when they were from areas showing "no-reflow." This is what one would expect constricted vessels to look like.

Krug et al. (68) reported a transient delayed reperfusion occurred in cat myocardium following 30 minutes of ischemia . This was not observed in dogs used in this study. In their experiment 6 of 12 animals had an extended inhibition of reflow following 60 minutes of ischemia. They describe these cats as having larger areas of risk than the 6 cats that had a shorter more transient inhibition of flow. One has to wonder if their 6 cats with smaller areas of risk are more like the dogs in this study and may also have had vasoconstrictions causing reflow inhibition. Grayson et al. (45) have suggested that vasospasms play an important role in determining the effect of vessel ligation on the myocardium. They were able to decrease the size of myocardial infarcts with the prior administration of adrenergic beta-blockers. They proposed that the release of catecholamines following vessel ligation could cause local vasoconstriction.

The transient inhibition of flow produced in this experiment is different from the previously reported myocardial no-reflow phenomenon. Firstly, it is not a permanent phenomenon. The present inhibition to reflow was gone from all but one sample by 30 minutes post vessel release. Secondly, unlike the phenomenon reported earlier by Darsee and Kloner (30) where the amount of tissue showing

this inhibition increased with time, here the percentage of severely ischemic tissue with this phenomenon declined. In the present study more samples with an ischemic period blood flow $\leq$ 25% of control have an inhibited reflow response after 60 minutes of occlusion than do so following 90 minutes and 120 minutes occlusions.

Several investigators (8,9,23,75) have used Evans blue dye to identify increased vascular permeability. Rawson (90) and Allen and Orahovats (1) were among the first to study the binding of Evans blue dye to plasma proteins primarily to albumin. They reported that at low concentrations Evans blue was completely bound to plasma proteins. Thus the presence of Evans blue dye extravascularly indicates that plasma proteins have escaped into the extravascular space. In the present experiment no samples with 30 minutes of ischemia had vascular leakage resulting in grossly visible Evan blue dye. Only 2 (6%) samples with a blood flow $\leq$ 25% of control flow for 60 minutes were stained. The percentage of samples served by vessels with increased permeability doubled following 90 minutes and further increased 3.6 fold after 120 minutes of similar ischemia. There was therefore a distinct increase in the number of samples served by leaking vessels with increasing length of ischemia. Schaper (101) has reported that the intensity of ischemia is responsible for increased vascular permeability. The present experiment demonstrates that the duration of ischemia is also important in the development of vascular damage. The failure of increased permeability to develop after 30 minutes of ischemia at the $\leq$ 25% of control flow level is further indication of the importance of the duration of ischemia on endothelial integrity.

It is well known that capillaries and venules are responsible for the leakage of proteins and fluids into the extravascular space following various insults. Capillaries and venules are most likely functionally damaged by ischemia due to

vessel occlusion higher up in the present experiment. Whether this damage is totally due directly to ischemia or is due to ischemia plus reflow was not determined in the present experiment. Increased permeability occurred almost exclusively in tissues from the most severely ischemic groups. A single sample ischemic for 2 hours with an ischemia period blood flow > 25 $\leq$ 50% of control flow did contain stain. The question that arises is whether longer periods of this degree of ischemia would increase the number of samples with stain in them. This investigator does not think this sample represents a trend in this direction. It is more likely that the focal blue stain present in part of this sample is due to a nonuniform drop in blood flow within the sample. Only vessels in tissue receiving $\leq$ 25% of control blood flow may have leaked. Flow in the rest of this sample probably was high enough to give an overall blood flow of > 25 $\leq$ 50% of control flow to the sample.

Schaper (101) has hypothesized that increased vascular permeability is the result of the actions of some "factor" released from ischemic myocytes rather than due to a direct effect of ischemia on vessel walls. The actions of this factor would have to last at least 20 minutes or the factor would have to resist being washed away by reflow. In the present experiment increased vascular permeability was still present following 20 minutes of reflow. Several authors (4,22,42,64,116) have previously demonstrated that ischemia produces numerous morphological changes in small myocardial vessels. It is not difficult to envision that widening of endothelial gaps and endothelial blebs, as described by these authors, is directly responsible for increased vascular permeability. Though the existence of such a "factor" as described by Schaper (101) cannot be disproved, it seems more likely that ischemia damages vessels directly.

In this study an attempt was made to keep the size of temporarily and permanently ischemic areas equal. No significant difference was found in the size

of these areas within each of the four groups studied. There was also a great deal of uniformity in the size of these areas among groups. Collateral circulation has been demonstrated as being a major determinant of the effects of acute ischemia on the myocardium (49,61,100,118). The collateral circulation of the heart in random bred non-related dogs should be quite variable from dog to dog. Therefore, if vessels supplying the same mass of tissue were ligated the effects of this action would more than likely be nonuniform. The same degree of ischemia would not be produced in all tissue at risk in every dog. An attempt was made in the present experiment to avoid this pitfall by ligating two vessels in the same dog. Thus each dog was its own permanent ischemic control. This is very important in any study seeking to compare the ultrastructural changes present in temporarily and permanently ischemic tissue. Wüsten, Flameng and Schaper (122) have reported that the amount of collateral circulation present in myocardium acutely ischemic is inversely related to the amount of tissue at risk. Ligation of large vessels endangers a large mass of tissue and the collateral supply to this tissue is limited. Ligation of a smaller vessel creates a smaller risk area that receives a larger supply of collateral flow with respect to the volume of tissue at risk. The degree of ischemia present throughout each of these areas will be quite variable. The pattern of blood flow in each of these areas following the termination of ischemia may also be quite variable. It was therefore necessary to use similar size ischemic areas in the same dog to overcome the confounding effects of collateral circulation.

Summary

This experiment has demonstrated that a transient inhibition to reflow can occur in the dog myocardium following temporary ischemia. Unlike previously reported in cats, this phenomenon does not occur following 30 minutes of temporary occlusion. It was found to occur in severely ischemic tissue following

60 minutes of ischemia and lessened after 90 and 120 minutes of ischemia. A second inhibition peak occurred in moderately ischemic tissue after 90 and 120 minutes of ischemia. Ischemia induced vascular permeability was shown to be related to both the degree and duration of ischemia. It was not however temporally related to the inhibition of reflow. Transient precapillary level vasoconstriction is most likely responsible for this phenomenon. The onset of this vasoconstriction was related to the degree of ischemia present in the tissue. If this is correct, patients with partially obstructed coronary vessels may be at greater risk of developing transient intramural myocardial precapillary spasms due to the effects of ischemia on these vessels. Finally, it has been demonstrated that following the termination of ischemia the reactive hyperemia response is initially greater in the subepicardium than in the subendocardium. However, 30 minutes after the start of reperfusion this relationship is reversed.

CHAPTER III

ULTRASTRUCTURAL CHANGES IN THE CORONARY MICROCIRCULATORY SYSTEM FOLLOWING ACUTE ISCHEMIA AND REFLOW

Introduction

Until recently little attention had been paid to ultrastructural changes occurring in the coronary microcirculatory system following myocardial ischemia. Jennings et al. (56) reported ultrastructural changes occurred in myocytes following acute ischemia in 1960. Since then there has been a voluminous amount of research done aimed at identifying the effects of ischemia on the myocardium. Most of these efforts have ignored the occurrence of changes in the ultrastructural morphology and function of small vessels supplying ischemic myocardium. Recently, several groups of investigators (4,22,42,64,116) have reported that morphological and functional changes in the coronary microcirculatory system do occur following ischemia. Morphological changes include focal and diffuse endothelial cell swelling, blebbing of endothelial cells, widening of endothelial cell gaps and focal to complete endothelial cell loss with microthrombi formation. Functional changes found have included increased vascular permeability and the possible obstruction of flow due possibly to a single or combination of morphological aberrations.

In this study morphological changes occurring in myocytes and coronary microcirculatory vessels following acute ischemia and reflow are evaluated. Since the dog is used as a model to study myocardial ischemia in humans, the investigator has compared the limited data present in the literature on the classification of normal human coronary microcirculatory vessels with his

observations in the dog. The classification of these vessels reported by Sherf et al. (105) served as the standard for the classification of vessels in the dog. Their work was done using biopsy samples from myocardium of patients undergoing cardiac surgery for a variety of reasons. Knowlege of any possible differences in the morphology of these vessels between these species is needed if the effects of ischemia on this vascular bed in the dog is to be useful in answering questions about its effects in man.

The purposes of this study were to: 1) identify the normal ultrastructural characteristics of coronary microcirculatory vessels in the dog and compare these with those of humans, 2) identify morphological changes in these vessels and myocytes following acute ischemia and reflow, and 3) evaluate the relationship between these changes and the transient inhibition of regional blood flow which occurs following release of a "critical" duration occlusion.

Methods

Experimental preparations: Forty healthy adult mongrel dogs of either sex, heartworm free and weighing 20-30 Kg, were used. These animals were fasted overnight and then sedated with 20 mg droperidol and 0.4 mg fetanyl (1 cc Innovar-vet). The surgical protocol has been previously described (Chapter II). Briefly, the dogs were anesthetized with halothane, nitrous oxide and oxygen and placed on a Harvard respirator following intubation. A left thoracotomy was performed, and a diagonal branch of the left anterior descending coronary artery and a marginal branch of the left circumflex coronary artery were isolated proximally (Figure 1). The two vessels were separated by a third vessel and appeared to serve an equal mass of tissue. A piece of umbilical tape strung through polyethylene tubing encircling each vessel and clamped with a hemostat was used to ligate each vessel.

Experimental design: Dogs were randomly assigned to one of four ischemia groups (Figure 2). The periods of ischemia were 30,60,90 and 120 minutes for

groups A-D, respectively. One vessel in each dog was temporarily occluded and the other permanently occluded for an equal period of time. Vessels were randomly chosen to be either temporarily or permanently occluded. All temporarily ischemic areas received 30 minutes of attempted reflow. Radioactive labeled 9 ± 1 μm microspheres were used to evaluate regional myocardial blood flow in these dogs. Microspheres were injected into the left atrium and a reference blood flow sample was withdrawn from the femoral artery at a rate of 7-10 ml per minute. Microspheres were labeled with either cerium-141 (Ce-141), chromium (Cr-51), strontium-85 (Sr-85), or scandium-46 (Sc-46). The order of microsphere injection was randomly determined.

Four separate microsphere injections were given to each dog. All dogs received a control period microsphere injection 5 minutes before ligation of the temporarily occluded vessels (Figure 2). Animals in group A received a second microsphere injection 10 minutes later to verify that ischemia had occurred in the area at risk. These animals were also given microspheres at the end of the temporary occlusion (start of the permanent occlusion) and at the end of the experiment. Animals in groups B-D received additional microsphere injections 40 minutes after the start of the temporary occlusion (10 minutes after the start of the permanent occlusion,), at the release of the temporary occlusion and at the end of the experiment. A 2% solution of Evans blue stain (Fischer Scientific), 1 ml/Kg, was given intravenously 5 minutes prior to the end of each experiment to study the effects of ischemia on endothelial integrity.

All hearts were stopped with KCl and were quickly perfusion fixed in situ. Hearts were perfused with a 0.9% saline solution via the ascending aorta at a perfusion pressure of 100-110 mm Hg followed by a 2% solution of phosphate buffered paraformaldehyde and glutaraldehyde 338 mOsm at the same pressure. The fixed hearts were then rapidly removed from the chest, blotted dry, weighed

and sliced from apex to base in transverse 5 mm sections (Figure 3A). Horizontal 1 mm thick transmural sections for transmission electron microscopy (TEM) were taken from the center of temporary and permanent ischemia zones and from normal areas from several slices (Figure 3B). These samples were further fixed at $4^{o}C$ for four hours in the same fixative solution used for perfusion. They were then placed in 0.15 M phosphate-sucrose buffer pH 7.4, 300 mOsM for several hours at $4^{o}C$. These tissues were then postfixed with osmium tetroxide dehydrated in graded concentrations of alcohol, embedded in Spurr epoxy resin, sectioned at silver grey interference color, mounted on naked copper grids, and then stained with uranyl acetate and lead citrate. Samples were then evaluated with a Philips 400 transmission electron microscope. Toluidine blue stained 1 μm sections were evaluated by light microscopy.

Heart slices were traced, photographed and the presence of Evans blue stain within slices was noted. Slices containing portions of either ischemia zone were divided into subepicardial, midwall and subendocardial samples (Figure 4). Radioactivity was determined with a Picker Autowell gamma counter and a model 4010, Pulse Height Analyzer. The size of each ischemia zone was determined using a blood flow of $\leq$ 50% of control blood flow to indicate severe ischemia.

Results

Experimental group: Of the 40 dogs that underwent surgery, 23 were used in this report . The other 17 were eliminated because of ventricular fibrillation (8 dogs); ischemic areas were too small to identify (6 dogs); significant scar tissue was identified in the heart at the time of surgery (2 dogs); and one set of microspheres was not given (1 dog). Groups A, B and D contained 6 dogs each and group C contained 5 dogs. Ventricular tachycardia developed in several dogs but was controlled with Lidocaine. There was no significant difference between

the mean size of the temporarily and permanently ischemia zone within each group using Student's t-test and a p value ≤ 0.05.

Arteriole: Arterioles ranged from 35-85 μm in diameter. They were lined by thin elongated endothelial cells which were 2 μm in width at the nucleus, and contained numerous cytoplasmic pinocytotic vessicles on both their luminal and abluminal membrane surfaces. Mitochondria were present, but not plentiful. Rough endoplasmic reticulum (RER) and a nucleus were the only other organelles consistently found in these cells. One to three layers of concentrically arranged smooth muscle cells composed the media in these vessels. The first layer of smooth muscle cells was separated from endothelial cells by the basement membrane of both cells, and a thin layer of amorphous matrix. Smooth muscle cells were 8-20 μm in length and 1-3 μm in width. Mitochondria were sparse relative to the large number present in cardiac myocytes. Other smooth muscle cell organelles included a large nucleus, dense bodies along the internal cell membrane and many myofibrils. Collagen, fibrocytes and nerve endings were a constant feature of the adventitial layer of these vessels. Collagen was also frequently seen between smooth muscle cell layers.

Precapillary sphincters: These vessels were 15-45 μm in diameter. They were lined by short, stumpy endothelial cells that were 3-5 μm in length and up to 3 μm in width (Figure 18). The nucleus in these cells was large, 2-3 μm in length and 2 μm in width, and bulged into the lumen. Pinocytic vesicles and RER were seen in the cytoplasm especially near the nucleus. These cells had a basement membrane on the abluminal surface. Finger-like projections of membrane bound cytoplasm extended through the amorphous matrix that separated endothelial cells and smooth muscle cells (Figure 19). These projections seemed to connect with similar projections originating from smooth

Figure 18 - Precapillary Sphincter. These vessels are 15-45 μm in diameter and are lined by stumpy, short endothelial cells that protude into the lumen. These vessels are surrounded by a single continuous layer of smooth muscle.
Bar = 10 μm.

Figure 19 - Myoepithelial Bridges in Precapillary Sphincters. Fingerlike cytoplasmic extensions, myoepithelial bridges (MB), extend from endothelial cells (E) and form junctions with similar projections from smooth muscle cells (SM). The basement membrane of endothelial cells and of smooth muscle cells appears to be continuous. Bar = 1 μm.

muscle cells in the media. The media was composed of a single continuous layer of smooth muscle cells whose basement membrane was continuous with that of endothelial cells. These cells averaged 21 μm in length and 4μm in width.

Metarterioles: Vessels in this category were 15–25 μm in diameter and some resembled precapillary sphincters (Figure 20). The endothelial cells lining these vessels bulged into the lumen but to a lesser extent than those lining precapillary sphincters. The nucleus in these cells nearly filled the cytoplasm. They were 4 μm in length and 1 μm in width. They, therefore, were not as thick as the endothelial cells in precapillary sphincters. Frequently the media consisted of a single complete layer of smooth muscle cells but more commonly this layer was only partially complete. Myoepithelial bridges were not as prominent as in precapillary sphincters. When present, they originated primarily from endothelial cells and were much shorter and blunted.

Capillaries: Capillaries were 4–8 μm in diameter and were lined by one to three thin endothelial cells (Figures 21,22). These endothelial cells were characterized by an abluminal surface basement membrane. Endothelial cell flaps, where one cell overlapped another, were prominent. Mitochondria were sparse, and with RER, were most common near the nucleus. The nucleus of these cells was large, 3 μm in length and 1 μm in width. However, they bulged into the extravascular space rather than into the lumen. Pinocytotic vesicles were more numerous in endothelial cells lining these vessels than in any other. These vessels did not contain any smooth muscle cells in their wall. They were, however, frequently surrounded by pericytes.

Venules: The diameter of these vessels ranged from 8-90 μm. They were lined by endothelial cells that contained flat elongated nuclei similar to those seen in arterioles. The smaller members of this group were not easily distinguished from capillaries. Pinocytotic vesicles were nearly as common as in capillaries. Smaller vessels were occasionally surrounded by pericytes. Smooth

Figure 20 - Metarterioles. These vessels are 15-25 μm in diameter and resemble precapillary sphincters. However, their endothelial nuclei are not as broad as those in precapillary sphincters and they do not bulge into the lumen as much. Furthermore, myoepithelial bridges are not prominent in these vessels and they are surrounded by a single discontinuous layer of smooth muscle.

Bar = 5 μm.

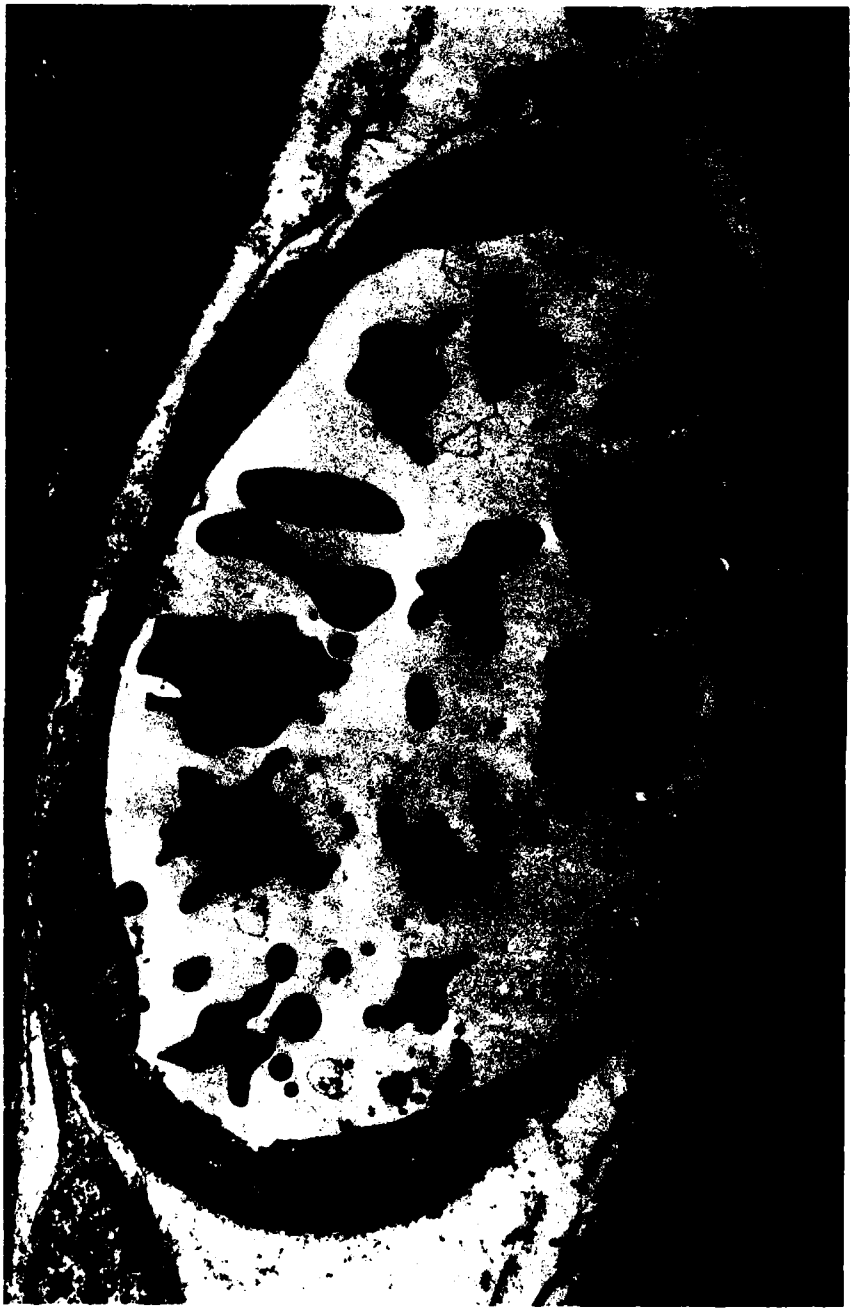
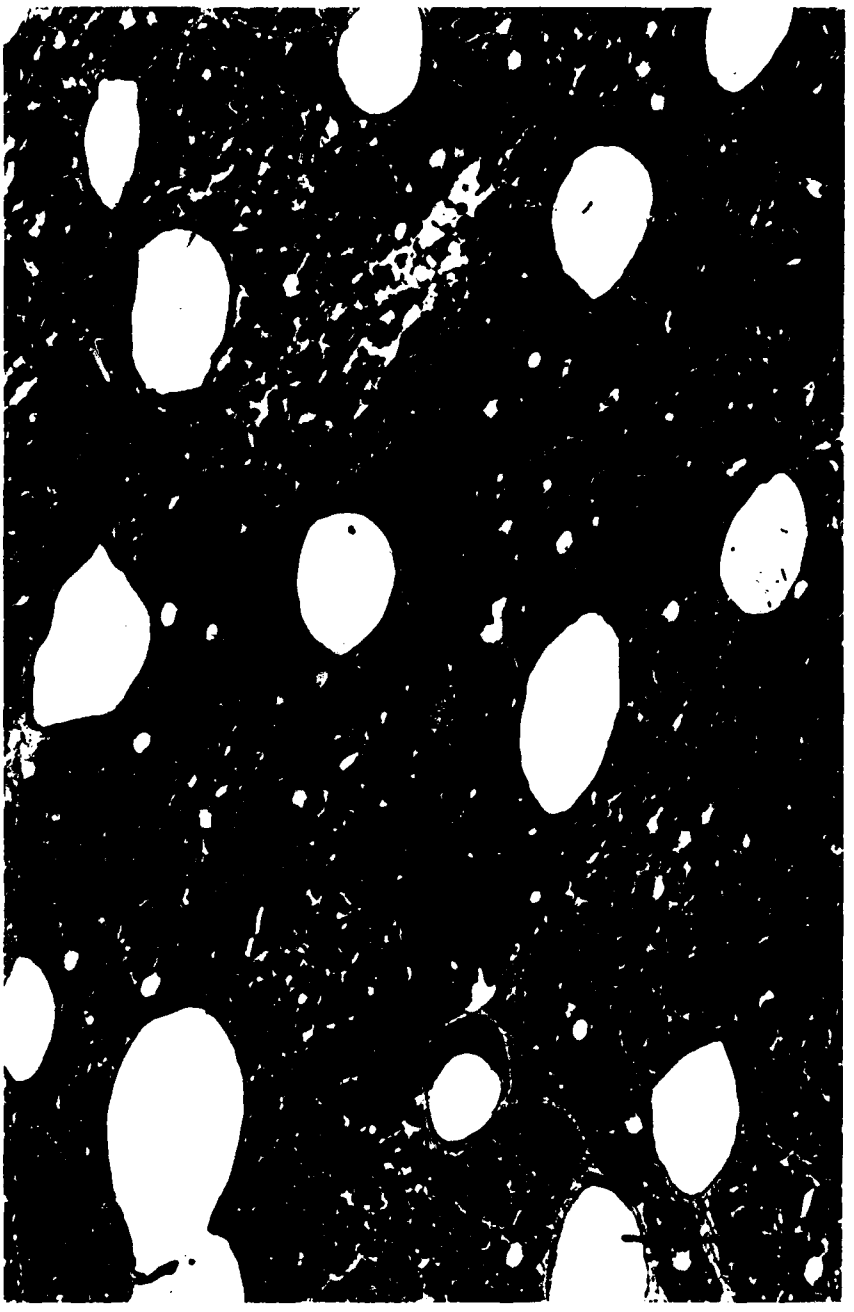


Figure 21 - Capillaries. Capillaries were 4-8 μm in diameter and were lined by 1-3 thin endothelial cells. These endothelial cells contained large nuclei that bulged toward the extravascular space.

Bar = 5 μm.



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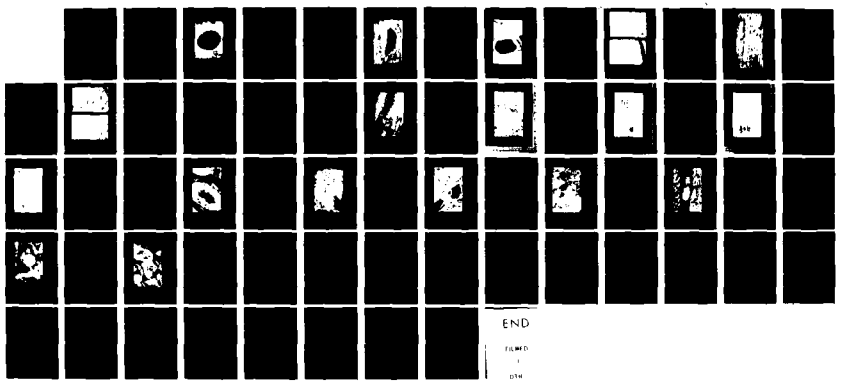

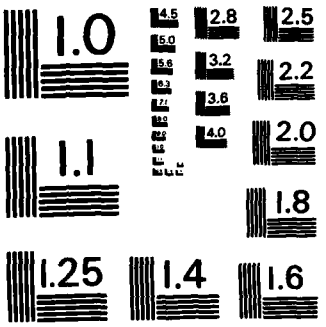

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Figure 22 - Capillary Surrounded by Pericytes. Frequently capillaries were surrounded by one or several pericytes (P). Note the large nucleus bulging toward the extravascular space and the endothelial flaps (EF) where endothelial cells overlap.

Bar = 1μm.


EF
P
P

muscle cells were rarely observed in the walls of these vessels; when present in the larger vessels, they did not form a complete layer. These vessels characteristically had large amounts of collagen intimately associated with their endothelial cell lining (Figures 23,24). Nerve endings were also frequently seen in close contact with these vessels, and along with collagen, they served as useful markers to distinguish small venules from capillaries.

Histology of non-ischemic tissue: The histological appearance of non-ischemic tissues was the same in all four ischemic groups. Myocytes were uniformly stained and rarely contained pale areas of cellular edema (Figure 25). Basophilic stained mitochondria were numerous throughout each cell and were in parallel arrangement with contractile elements. Nuclear chromatin was well dispersed and clumping or margination was not seen. Little extracellular space was present between myocytes. Numerous capillaries were interspersed between myocytes.

Histological alterations in ischemic tissue: After 30 minutes of severe permanent ischemia, mild morphological alterations were detected in subendocardial samples receiving $\leq$25% of their control blood flow during ischemia. Lesions in the subepicardium were quite subtle. They included mild perinuclear edema and mild to moderate chromatin margination (Figure 26). Capillaries in these tissues were frequently filled with red blood cells indicating these samples were not perfused during perfusion fixation. An increase in interstitial space was not observed in these samples.

Morphological changes were most prominent in subendocardial samples. Myocytes in these samples were more edematous than in epicardial samples and their myofibrils often appeared moderately separated (Figure 27). Nuclear chromatin margination was common in these samples. Red blood cells often filled the lumens of small vessels in subendocardial tissues. Changes in the

Figure 23 - Venules. These vessels were 8-90 μm in diameter and were lined by thin endothelial cells. The nucleus in these cells were long and thin. Collagen (C) usually surrounded these vessels.

Bar = 1μm.


C
C

Figure 24 - Venules Surrounded by Collagen. Venules were often surrounded by broad bands of collagen (C).

Bar = 5μm.

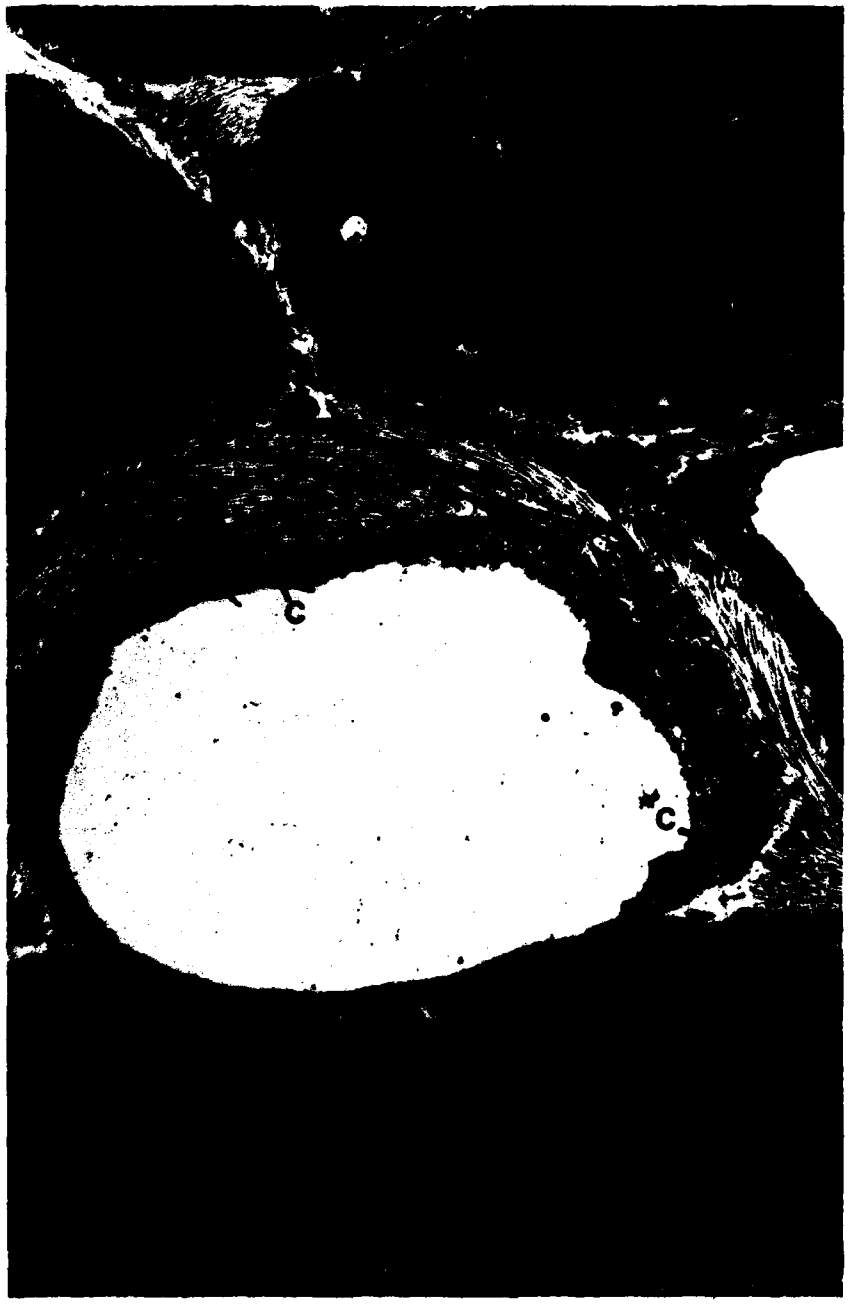

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Figure 25 - Normal Myocyte Histology. Myocytes were evenly stained and contained numerous mitochondria (arrows). Cellular edema was not observed. Dilated capillaries are interspersed between myocytes.
Bar = 10μm.

Figure 26 - Histology of Subepicardium Following 30 Minutes of Permanent Ischemia. Mild perinuclear edema and pale nuclei due to chromatin margination.
Bar = 10μm.

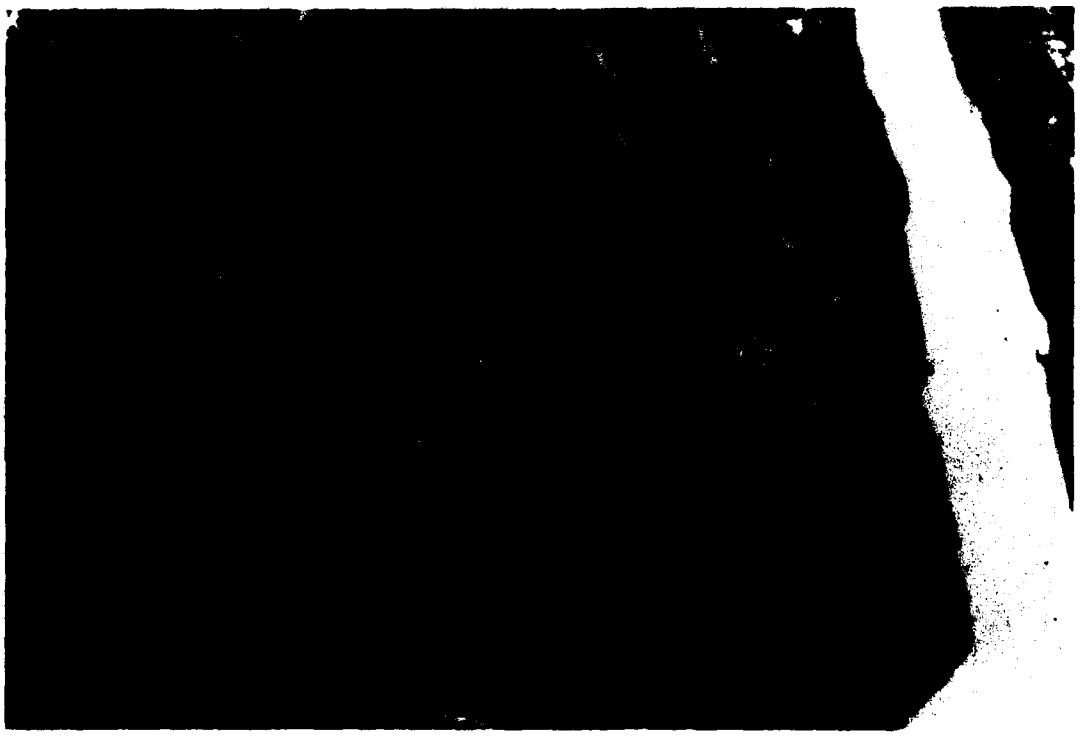

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Figure 27 - Histology of Subendocardium Following 30 Minutes of Permanent Ischemia. Nuclear chromatin margination and pale myocytes were common. Capillaries were frequently occluded with red blood cells (arrows).

Bar = 10μm.

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midwall were intermediate between those in the subepicardium and the subendocardium. Ischemia induced alterations were widely spread through subendocardium samples. However, edematous cells with nuclei containing marginated chromatin were frequently seen next to cells that appeared normal. A thin band of myocytes just beneath the endocardium was routinely spared. These cells are thought to be nourished by diffusion of metabolites from the ventricular lumen.

Reperfusion following 30 minutes of ischemia caused a decrease in myocyte edema and a dispersion of nuclear chromatin. Though reperfusion appeared to reverse ischemic induced alterations, this reversal was incomplete. Mild cellular edema was present in myocytes even after reperfusion. Frequently, however, it was difficult to distinguish non-ischemic from reperfused samples.

Following 60 minutes of permanent ischemia with $\leq$ 25 percent of normal blood flow tissues were only slightly more damaged than those subjected to 30 minutes of ischemia. They were slightly more edematous and contained more nuclei with marginated chromatin. Capillaries and venules not filled with red blood cells appeared to be widely patent. Tissues which were reperfused were drastically different from those which were permanently ischemic. Reperfused myocytes were moderately edematous, failed to stain as deeply as their non-reperfused counterparts, and their myofibrils were often separated (Figure 28). Dark basophilic wavy lines (contraction bands) frequently extended transversely across these myocytes (Figure 28). This change was most commonly seen in myocytes whose myofibrils appeared stretched and were separated by edema. These contraction bands have been previously described by numerous investigators. They represent supercontraction and degeneration of contractile elements. Nuclei in reperfused myocytes contained severely marginated chromatin and often appeared quite pale. Islands of apparently normal cells were

Figure 28 - Reperfused Myocytes Following 60 Minutes of Ischemia. Contraction bands (CB) were first observed in this group.

Bar = 10μm.

Figure 29 - Reperfused Myocytes Following 90 Minutes of Ischemia. Contraction bands (CB) were more prominent in these samples than in those ischemic for 60 minutes. Myocytes were often pale and myofibrils were disorganized (arrows).

Bar = 10μm.

sometime surrounded by severely damaged cells. While interstitial edema was not seen in tissues reperfused after 30 minutes of ischemia, it was present in tissues reperfused after 60 minutes of ischemia. Microthrombi were not seen in samples from any of the four ischemia groups. In fact, vessel lumens consistently appeared histologically patent.

Lesions present after 90 and 120 minutes of permanent ischemia were not significantly different from those present after 60 minutes. Samples permanently ischemic for 120 minutes were slightly more edematous and contained more nuclei with marginated chromatin. Reperfusion after 90 minutes of ischemia increased the number of contraction bands and the level of cellular and interstitial edema (Figure 29). Myofibrils in these cells were often quite disorganized. These same changes were present after reperfusion following 120 minutes of ischemia. They were, however, more prominent and few islands of normal cells were observed.

Ultrastructural alterations: As was observed histologically, ultrastructurally there existed a transmural gradient in the severity of lesions present in tissue samples. This was true, even when subepicardial and subendocardial samples were subjected to comparable levels of ischemia. Ultrastructural morphologic alterations were graded according to a modified version of the grading system used by Gottlieb, Kubo and Alonso (44). Myocyte changes which were evaluated included:

1) glycogen loss: The occurrence of decreased glycogen within myocytes was graded as mild, moderate or severe. A mild loss was one recognized only with difficulty and consisted of mild paleness around nuclei. A moderate loss was more easily recognized and consisted of large pale areas perinuclearly and around mitochondria. Severe loss occurred if these and other areas were almost devoid of glycogen.

2) sarcoplasmic edema: Subsarcolemmal blebs (1+) to 50% or greater cell volume expansion (4+).

3) mitochondrial swelling: Mild separation of cristae (1+) to greater than doubling of mitochondrial volume (4+).

4) mitochondrial densities: Rare granular densities present (1+) to large densities present in most mitochondria (4+).

5) nuclear chromatin clumping: Slight granular clumping (1+) to marked coarse clumping and extreme margination (4+).

6) contraction band: Focal hypercontracted myofibrils involving several sarcomeres, with attenuation of myofibrillar elements on either side of this area (1+) to the presence of these changes in nearly every cell (4+).

7) lipid droplets: Occassional or rare (1+) to numerous in most cells (4+).

Microcirculatory vessel alterations were graded using the same criteria where applicable. Other parameters evaluated in these structure included:

1) endothelial cell edema: Few vessels lined by focally swollen endothelial cells (1+) to more than half being more than 50% occluded (4+).

2) endothelial cell loss or gap formation: Focal widening of endothelial cell junctions (1+) to a total loss of endothelial cells (4+).

3) platelet plugs and microthrombi: A few vessels containing platelet plugs or microthrombi that do not obstruct more than 50% of their lumen (1+) to more than half of these vessels being more than 50% occluded (4+).

Scores were assigned while samples were being viewed with the electron microscope. Since no attempt was made to grade electron micrographs of

representative areas, statistical quantitation of lesions was not done. Grading was done to facilitate the recognition of trends in lesion occurrence and not to quantitate their presence.

Thirty minutes of ischemia: Ultrastructural changes following 30 minutes of permanent ischemia were easily recognized. Glycogen loss was mild to moderate and sarcoplasmic edema was characterized primarily by subsarcolemmal blebs. However, large foci of edema were also occassionally seen. Minimally affected cells were frequently surrounded by cells that were moderately to severely damaged (Figure 30). In more severely affected areas sarcoplasmic edema caused cells to be at least 25% increased in volume. Mitochondria in these cells were usually comparably swollen (Figure 31). Mitochondrial granular densities were only occasionally seen. Myofibrils in these cells were widely separated by edema and were severely contracted (Figure 31). Contraction bands were not seen in any permanently occluded samples from any ischemia group. Mild to severe coarse clumping of nuclear chromatin was present in a majority of myocytes (Figure 31). Lipid droplets were present in most myocytes, though sometimes they were seen only after considerable searching.

Endothelial cells in capillaries and venules were usually either normal or were focally, mildly swollen. However, some contained endothelial cell blebs that partially or nearly completely obstructed the vessel lumen (Figure 32,33). Less than 5% of the capillaries and venules present in these sections were more than 50% occluded. Mitochondria in severely affected endothelial cells were often quite swollen. Large vacuoles, often present in the cytoplasm of these cells, may have been formed from degenerating mitochondria (Figure 34). Endothelial cell nuclei in extensively damaged vessels were shrunken and contained clumped chromatin (Figure 34). Larger vessels with smooth muscle cells in their wall were only minimally damaged. Mitochondria in endothelial and

Figure 30 - Nonuniform Cell Injury Following 30 Minutes of Ischemia. Minimally affected cells (M) often were adjacent to more severely injured cells (S). Glycogen loss and intermyofibrillar edema was more prominent in severely affected cells. Subsarcolemmal blebs (B) and endothelial swelling (ES) were also present at this time.

Bar = 1μm.


ES
ES
S

Figure 31 - Severely Injured Myocytes After 30 Minutes of Ischemia. The nuclei in these cells were often pale due to marginated and clumped chromatin. Cell edema was present in these cells, but the amount seen was highly variable. Mitochondria were mildly swollen and contained separated cristae.
Bar = 1μm.

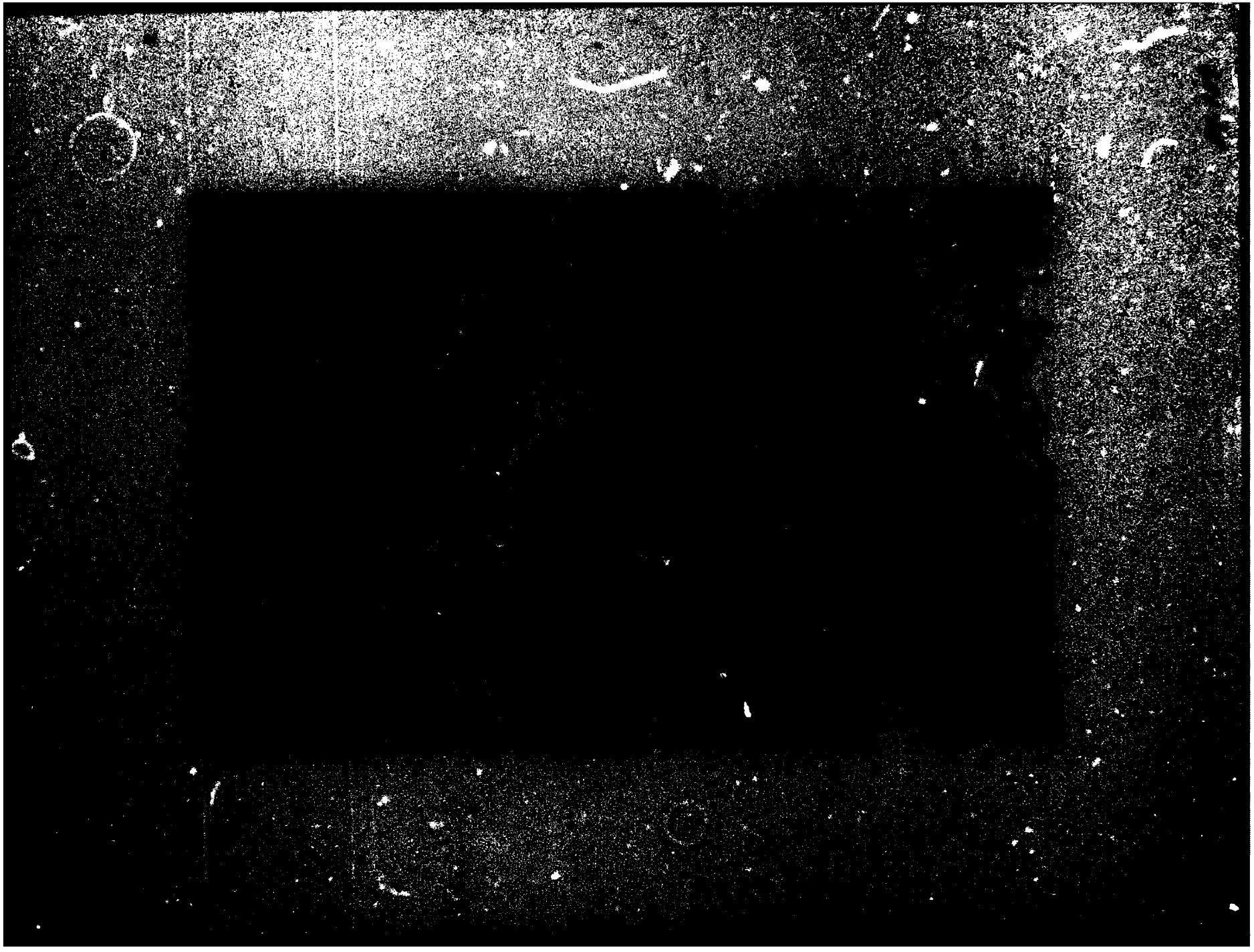

Figure 32 - Capillary Endothelial Blebs and Myocyte Subsarcolemmal Blebs. Capillary endothelial blebs (EB) frequently partially occluded these vessels. Blebs were found still attached to the endothelial wall and floating free within capillary lumen. Subsarcolemmal blebs (SB) were not usually associated with a stenosed vessel.

Bar = 1μm.

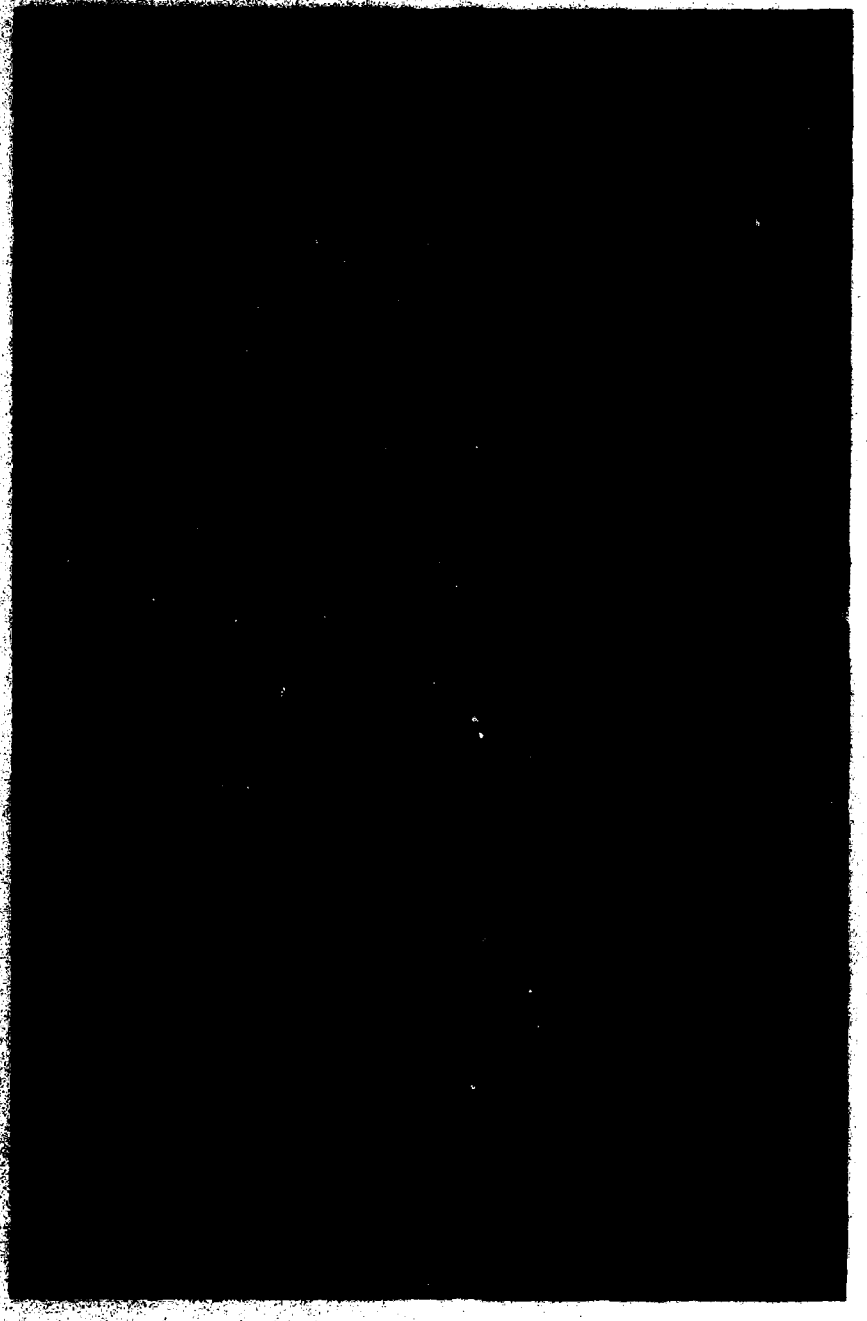

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Figure 33 - Capillary Stenosis Due to Endothelial Blebs. Less than 5% of the capillaries and venules observed appeared to be more than 50% occluded. Endothelial blebs (EB) and endothelial swelling were the main cause of occlusion in these vessels. A red blood cell (RBC) seems barely able to traverse this vessel.

Bar = 1μm.

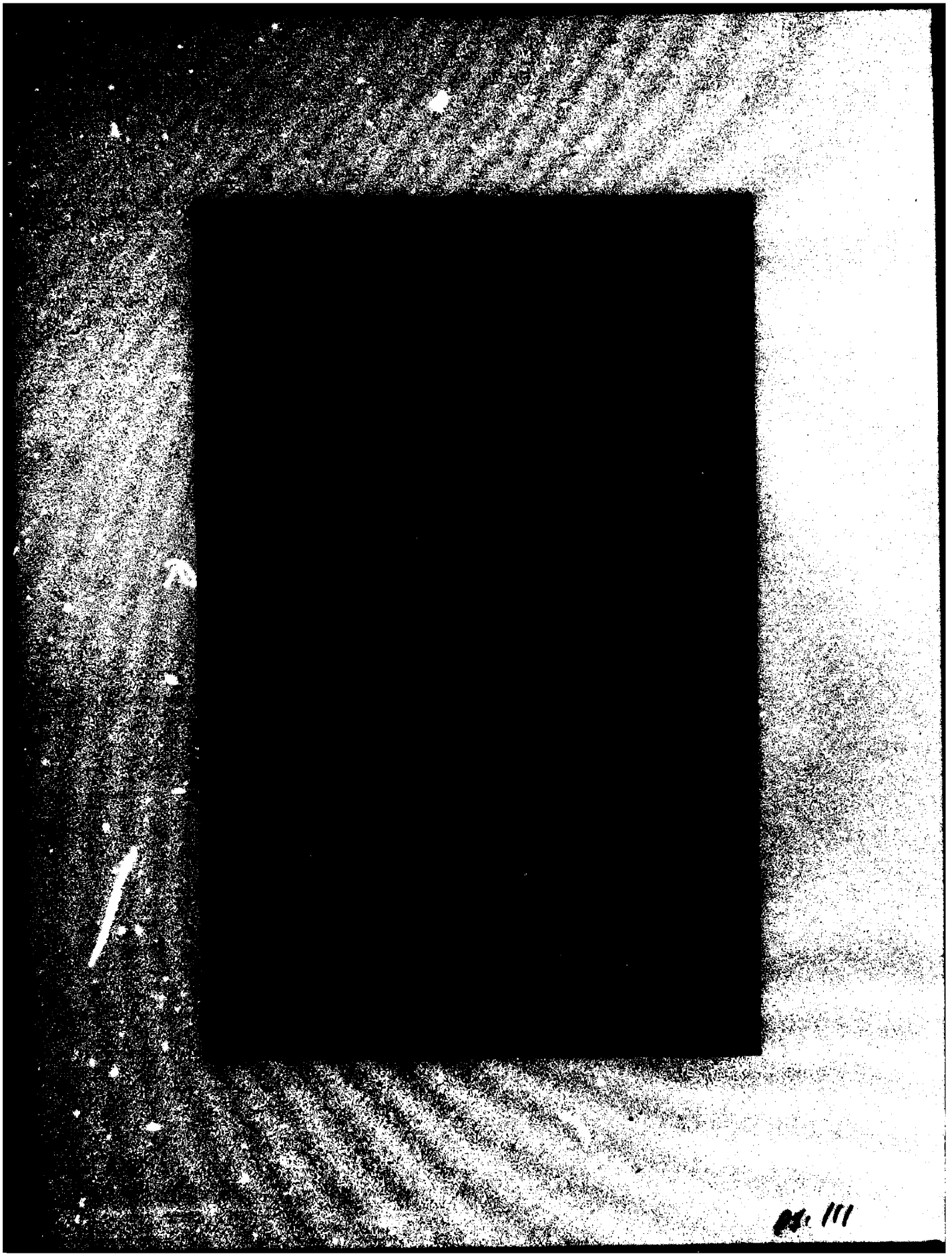
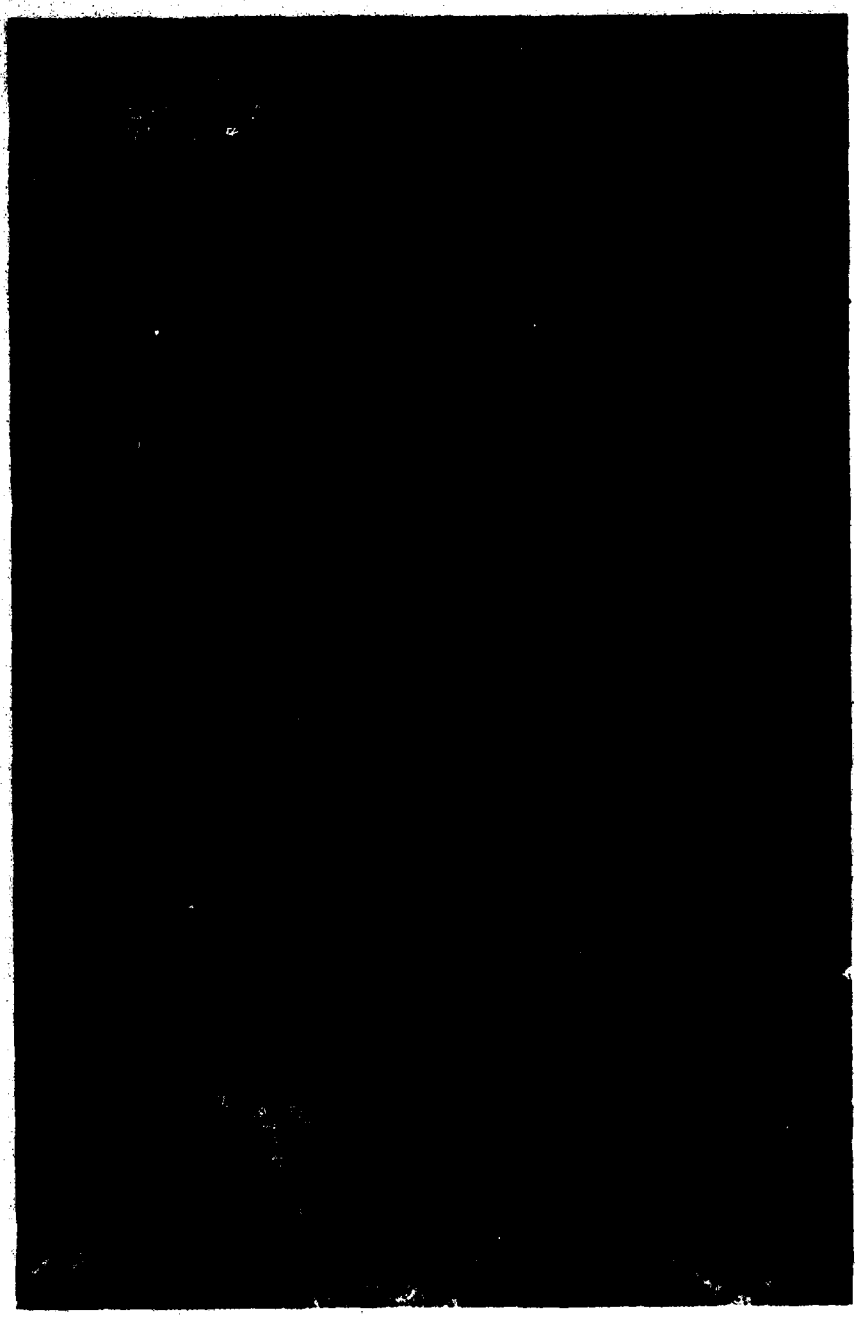


Figure 34 - Endothelial Mitochondrial Degeneration. Mitochondria (M) in endothelial cells of affected vessels were often ballooned and vacuolated. The nucleus in these endothelial cells were sometimes contracted and dark. Damaged vessels frequently were surrounded by myocytes that were minimally affected and contained mildly vacuolated mitochondria (MM).

Bar = 1μm.



smooth muscle cells in these vessels were moderately swollen and contained a pale matrix (Figures 35 and 36). Glycogen was less prominent in these cells as in endothelial cells lining smaller vessels.

After reperfusion, severely ischemic samples were less damaged than their non-reperfused counterparts. Lipid droplets, though still present were less commonly seen. Glycogen was now present in nearly normal quantities. Mitochondria contained tightly packed cristae and rare dense granular inclusions (Figure 37). Perimitochondrial edema and subsarcolemmal blebs were still present, but less severe. Reperfusion for 30 minutes had not detectably altered the appearance of endothelial cells in capillaries and venules. However, nuclear chromatin did appear to be less marginated in the nuclei of these cells.

Sixty minutes of ischemia: 60 minutes of permanent ischemia caused more severe myocyte lesions. These cells were more edematous and contained more severely damaged mitochondria. Intramyofibrillar edema was much more common in these cells than in those receiving 30 minutes of ischemia. Mitochondria commonly contained widely dispersed tubular cristae and fragmented mitochondrial membranes (Figure 38). Intramitochondrial dense granular inclusions were more common in these samples than in those made ischemic for 30 minutes, and in those subsequently reperfused. Myocyte nuclear chromatin was increasingly clumped and marginated. Glycogen was more severely reduced, but was never totally absent. Lipid droplets were more common in these tissues than in previously described ones. Microcirculatory vessels were no more damaged than in tissues ischemic for 30 minutes. However, widening of endothelial cell junctions was first identified in these samples (Figure 39).

Reperfusion of tissues in the 60 minute ischemia group resulted in more severe myocyte lesions. Large numbers of super contracted myofibrils and

Figure 35 - Ischemic Precapillary Sphincter. The endothelial and smooth muscle layers of these vessels were minimally affected by ischemia. Mitochondrial vacuolization (M) was often the only change seen in the cells of these layers. Bar = 10μm.

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Figure 36 - Ischemic Precapillary Sphincter. Higher magnification. Higher magnification of part of precapillary sphincter wall showing mitochondrial vacuolization (M) but relatively normal endothelium and smooth muscle cell nuclei.

Bar = 5μm.

Figure 37 - Reperfusion Following 30 Minutes of Ischemia. Following reperfusion severely ischemic samples were less damaged than their non-reperfused counterparts. Mitochondria were less vacuolated and contained tightly packed cristae. Subsarcolemmal blebs were less prominent in myocytes and their nuclear chromatin was less marginated.

Bar = 1μm.

Figure 38 - Sixty Minutes of Permanent Ischemia. Mitochondria in myocytes ischemic for 60 minutes were often severely swollen, contained separated fragmented cristae and were surrounded by a ruptured mitochondria membrane (MM).

Bar = 1μm.

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Figure 39 - Widened Endothelial Cell Gaps. After 60 minutes of ischemia widened endothelial cell gaps (G) was first observed in capillaries and venules. This vessel contains a contracted dark nucleus, pale swollen endothelium and a red blood cell in its lumen. Surrounding myocytes are also severely damaged. Bar = 1μm.

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contraction bands were present in these samples (Figure 40). Myocytes were quite edematous. Cellular swelling increased myocyte cell volume by approximately 40%. Mitochondria in these cells were less swollen than those in nonreperfused samples, but they contained a larger number of intramitochondrial granular densities (Figure 39). Microcirculatory vessels were unaffected by reperfusion. Mitochondria in endothelial cells and smooth muscle cells rarely contained densities. Less than 10% of the capillaries and venules in these reperfused samples were 50% occluded by swollen endothelial cells or endothelial cell blebs. Subsarcolemmal blebs rarely appeared to be capable of causing functional stenosis of microcirculatory vessels. The vast majority of capillaries and venules were lined by endothelial cells that were only focally swollen. Precapillary sphincters, arterioles and metarterioles were basically unaffected by reperfusion. Mitochondria in their endothelial and smooth muscle cells looked like those in capillary and venous endothelium.

Ninety and one hundred twenty minutes of ischemia: After 90 and 120 minutes of permanent ischemia it was difficult to detect an increase in myocyte damage greater than that which was present following 60 minutes of ischemia. However, severely damaged cells were more uniformly present throughout these samples. This was especially apparent following reperfusion. Ruptured mitochondria, contraction bands, and intramyofibrillar edema were all more common in these reperfused samples than in those in other ischemic groups. Intramitochondrial granular densities were no more common in these samples than in samples reperfused after 60 minutes. In fact, following 120 minutes of ischemia and reperfusion, the number of these densities had actually declined. Though mitochondria contained fewer densities, their cristae were often tubular, fragmented and angular (Figure 41). Widened endothelial cell gaps were most common in tissues ischemic for 120 minutes. Roughly 15% of the capillaries and

Figure 40 - Reperfusion after sixty minutes of ischemia. Reperfusion after 60 minutes caused contraction band (CB) formation and more severe intermyofibrillar edema. Dense mitochondrial inclusions (DMI) were frequently seen in the mitochondria of the samples.

Bar = 1μm.

Figure 41 - Reperfusion After 90 Minutes of Ischemia. Though dense mitochondrial inclusions (DMI) were seen in these samples, they were no more common than in samples reperfused after 60 minutes. Tubular mitochondria cristae were prominent in these samples.

Bar = 1μm.

Fig. 129

venules in these samples contained these gaps. Reperfusion of these tissues did not increase the occurrence of gaps.

Postischemic blood flow and ultrastructural change: Following release of the temporary occlusion, an inhibition of immediate reflow was detected in some samples (Figures 13-16). As previously stated, this inhibition was a transient phenomenon. It was most commonly seen in tissues severely ischemic for 60 minutes. No direct relationship between reflow inhibition and ultrastructural alterations was seen. Though severely ischemic samples from Group B demonstrated the most severe inhibition of reflow, ultrastructural damage was uniformly more severe following 120 minutes of ischemia and reperfusion.

Vascular permeability and endothelial cell alteration: Evans blue dye was not grossly visible in any tissues subjected to 30 minutes of ischemia and reperfusion (Figure 5). Endothelial cells in 5% of the capillaries and venules in these samples occassionally contained numerous blebs or were so swollen they were functionally occluded (Figure 32,33,34). Degenerative large mitochondrial vacuoles present in the cytoplasm of these cells were further indication that these cells were indeed injured (Figure 33). Widening of endothelial cell junctions was not seen in these vessels. After 60 minutes of ischemia only 2 (6%) of 36 temporarily ischemic samples contained blue stain (Figure 5). The ultrastructure of these vessels was similar to that of vessels ischemic for 30 minutes. Endothelial cell junctions in these vessels were however, occasionally widened (Figure 37). Following 90 and 120 minutes of temporary ischemia 4 of 32 (12%) and 17 of 42 (40%) of these tissues contained stain, respectively (Figure 5). The number of capillaries and venules that were more than 50% occluded never exceeded 10% in these samples. Widening of endothelial cell junctions in capillaries and venules was most common after 120 minutes of ischemia.

Discussion

The ultrastructural morphology of the microcirculatory vessel in the dog is only slightly different from that described by Sherf et al. (105) for these vessels in man. Somewhat surprising was the fact that the diameter of vessels in our perfusion fixed samples was close to values they reported for these vessels in immersion fixed biopsy samples.

Sherf et al. (105) reported that arterioles were 30-100 μm in diameter, whereas vessels of similar morphology in our samples were 35-85 μm in diameter. In both studies, these vessels were lined by thin endothelial cells with flat, elongated, nonbulging nuclei. One or three layers of smooth muscle cells were found in the walls of these vessels and collagen, fibroblasts and nerve elements were seen in their adventitia.

Precapillary sphincters have been described as vessels 15-30 μm in diameter, lined by endothelial cells whose nuclei protrude into their lumen. The media in these vessels contain one complete layer of smooth muscle in their wall (105). The diameter of these vessels in our samples was 15-45 μm. Fawcett (33), Rhodin (93) and Sherf et al. (105) have suggested that constriction of these vessels produces a close approximation of the large endothelial nuclei lining these vessels. This would lead to functional occlusion and subsequent cessation of distal flow. Membrane bound cytoplasmic projections in these vessels, myoepithelial bridges, have been suggested to be a thoroughfare for the movement of blood borne transmitter substances (33,93,105). These substances are thought to bind to endothelial cell receptors and then are transferred across myoepithelial connections to smooth muscle cells that then constrict or relax. These investigators (33,93,105), as well as Harris and Longnecker (47) have proposed precapillary sphincters are the vessels responsible for controlling capillary flow. Schaper and Schaper (99) remain skeptical of the existence of

precapillary sphincters in the heart, though they acknowledge their presence in other organs. They have suggested that intermittent capillary flow may be due to arteriolar activity. In the work of Sherf et al. (105) a photomicrograph depicting the occlusion of a vessel by its bulging nuclei is presented. No similar pattern of vessel occlusion was observed in the perfusion fixed material of the present experiment. However, it is not difficult to envision that the bulging nuclei in our samples could partially or completely inhibit blood flow when these vessels are constricted.

Vessels classified as metarterioles in the present report were morphologically somewhat different from those previously described for man (105). The diameter of these vessels (15-25 μm) was similar to that previously reported (10-20 μm), but the appearance of their endothelial cells was distinctly different. In man the endothelium lining these vessels is "sponge-like" in appearance (105). This appearance is due primarily to thin villous projections arising from endothelial cell membranes and extending into the lumen (103). Cytoplasmic vacuoles, several times larger than pinocytotic vessicles, also help create the "sponge-like" appearance of this layer (105). The endothelium lining these vessels in the dog do not contain villi or large cytoplasmic vacuoles. These vessels in the dog, like those in man, contain either a single complete layer of smooth muscle in their walls or an incomplete layer. Delayed immersion fixation may cause endothelial cells to be thinner than normal and may also cause endothelial cell bleb formation and vessiculation (99). Endothelial and smooth muscle cells from ischemic tissues in the present experiment frequently contain vacuoles, thought to be degenerative mitochondria; whether or not the "sponge-like" appearance of the endothelium in these vessels, as reported by Sherf et al. (105), is the result of autolysis associated with delayed immersion fixation or is a species difference is not known.

Capillaries in human myocardium have been reported to be 5-8 μm in diameter (105). They have endothelial villi, large cytoplasmic vascuoles and their large nuclei bulge extravascularly (105). In non-ischemic dog myocardium the endothelium lining these vessels does not possess villi nor contain large vacuoles. Venules in dog myocardium were slightly different from those seen in man. These vessels did not have villi or large vacuoles associated with their endothelium. In the dog, these vessels were closely associated with nerve endings. Mild to moderate amounts of collagen were present in the adventitia of these vessels in man and the dog (105).

Thirty minutes of permanent ischemia produced histological alterations in myocytes and ultrastructural alterations in both myocytes and microcirculatory vessels. The histological changes observed in these tissues were subtle and similar to early autolytic changes. These changes included perinuclear edema and nuclear chromatin margination. The absence of these changes in nonischemic tissues and their being less prominent in reperfused tissues indicate they are not autolytic in origin. Histological alterations in subendocardial samples were consistently more severe than they were in subepicardial samples. This was true even when both had been subjected to equal or nearly equal ischemia. Reimer et al. (92) first reported that this occurred and labeled this the "wavefront phenomenon." They showed that ultrastructural alterations due to ischemia, started first in the subendocardium and then progressed across the wall toward the subepicardium in a wave-like fashion. Reperfusion following 30 minutes of temporary ischemia was histologically beneficial in the present study.

After 60 minutes of permanent ischemia, myocytes were more edematous, intramyofibrillar edema was present and more nuclei contained clumped and marginated chromatin. Reperfusion was detrimental rather than beneficial in this group. Myofibrillar edema and disorganization were made much worse by reperfusion and contraction bands were now evident. Mild interstitial edema was

now mildly apparent. It was difficult to determine if 90 or 120 minutes of permanent ischemia caused more severe lesions than 60 minutes of ischemia. The most severe lesions seen after 60 minutes of ischemia were more uniformly present in tissue samples with 90 or 120 minutes of ischemia. However, even after 120 minutes of ischemia with reperfusion, not all cells were equally affected. Reperfusion did drastically increase the number of contraction bands and the amount of myofibrillar fragmentation present in the 120 minute samples. Microcirculatory vessels in all four ischemic groups were histologically unaffected by ischemia and by reperfusion.

The "wavefront phenomenon" was also observed ultrastructurally in ischemic tissues. Subepicardial samples, permanently and temporarily ischemic, were less severely affected than were comparably ischemic corresponding subendocardial samples. Bell and Fox (10) have reported that though the subendocardium is normally perfused at a slightly higher rate than the subepicardium, it exists in a state of relative hypoxia. This is due to its being perfused primarily only during diastole and because systolic tension in the subendocardium is often equal to peak systolic pressure (10). Therefore, these borderline hypoxic myocytes have to work harder. Ischemia is therefore more critical initially in these fibers. Over a period of six hours, structural changes have been found to move like a wave toward the subepicardium. After this time, all fibers that will die due to an ischemic insult are irreversibly injured (92). Irreversible injury has been reported to be present when intramitochondrial granular densities, mitochondrial fragmentation and contraction bands occur (56,101). Intramitochondrial granular densities have been reported to be aggregates of Ca PO_4 and they are often the earliest indicators of irreversible injury (64,103,104). Contraction bands are thought to be formed when irreversibly injured myocytes are exposed to calcium

via reperfusion. Contractile elements in these cells supercontract and myofibrillar material on either side becomes stretched, fragmented and separated.

Comparisons of temporarily and permanently ischemic samples were made in this study without the confounding effects of different levels of collateral supply. This was possible because there was no significant difference between the size of temporarily and permanently ischemic areas within groups. Since temporarily and permanently ischemic areas from the same dog were compared, the problem of genetic variation in collateral supply was also circumvented. The importance of collateral blood flow as a determinant of the myocardium's response to ischemia is well documented (95,101,122). The collateral supply to a given mass of tissue varies inversely with the volume of tissue at risk (122). The larger the mass of tissue at risk, the smaller the surface area to volume ratio. Therefore, fewer collateral vessels can impact on the surface of a large mass of tissue as opposed to a smaller one.

Ultrastructural myocyte alterations in this study were similar to those previously reported (49,56,63,64,65,101). Intramitochondrial granular densities were rarely seen after 30 minutes of permanent ischemia. These densities were more common following 60 minutes of permanent ischemia. Mitochondria in tissues reperfused after 60 minutes of ischemia contained even more of these densities. Mitochondria in tissues ischemic for 90 and 120 minutes, temporarily and permanently, contained fewer intramitochondrial densities than did those in tissues subjected to 60 minutes of ischemia and reperfusion. Kloner et al. (64) have reported these densities were not observed in their tissues, which had been ischemic for 90 minutes and then allowed reflow. They suggested that mitochondria in these cells had lost their ability to accumulate calcium. A similar phenomenon has been observed in mitochondria studied in vitro (64).

Biochemical proof of a loss in calcium uptake ability in these mitochondria does not yet exist. However, data in the present experiment supports this hypothesis.

Reperfusion following 30 minutes of ischemia was beneficial to myocytes. Changes present in these cells due to permanent ischemia were either absent or were greatly reduced following 30 minutes of reperfusion. Reperfusion after 60, 90, and 120 minutes of ischemia caused myocytes to be more severely damaged. Contraction bands were commonly seen in these cells, along with increasingly common more severe nuclear chromatin clumping and margination. These cells, unlike those in samples ischemic for 30 minutes, were irreversibly injured. Jennings et al. (57) have reported that following as little as 20 minutes of ischemia, some myocytes are irreversibly injured. In this experiment the time at which irreversible injury occurs is probably a function of heart rate, the anesthetic used, size of the area at risk and the collateral supply to this area.

Several investigators (4,42,64,116) have reported coronary microcirulatory vessels are damaged by ischemia. Armiger and Gavin (4) have reported that though marked endothelial swelling was seen following 10-60 minutes of ischemia, not all endothelial cells were simarly affected even after 120 minutes of ischemia. They further reported that endothelial cell swelling may occlude the capillary lumen. A reassessment of these findings by Gavin et al. (42) led them to suggest that reflow inhibition is probably not due to endothelial cell swelling or capillary occlusion of any nature. Though endothelial cell swelling was observed in their capillaries and venules, most vessels were dilated rather than compressed when vascular competence was lost. Kloner et al. (64) have also reported that capillary damage was a major factor in reflow inhibition observed in their samples.

Endothelial cells lining most capillaries and venules were focally swollen after 90 minutes of ischemia. Reperfusion for 30 minutes following either 30, 60, 90 or 120 minutes of ischemia does not alter the appearance of these vessels.

Histologically, capillaries and venules in all four ischemia groups appeared normal. Ultrasturally however, focal edema, blebbing and even complete occlusion was seen. No more than 10% of the capillaries and venules present in tissue from all ischemic groups was ever observed to be more than 50% occluded. This finding, along with the report by Gavin et al. (42), indicates that capillary occlusion is not a major determinant in reflow inhibition in postischemic myocardium. Capillaries and venules in tissue with an inhibited initial reflow were no more occluded than those with an initial reactive hyperemia flow three times greater than control. Red and white blood cells packing within capillaries, microthrombosis and parenchymal cell edema have been suggested to be important in inhibiting reflow (3,4,27,32,64). In the present study most of these factors were rarely, if ever, present and therefore played no major role in causing the transient inhibition of flow which was observed. Red blood cell packing was frequently seen in permanently occluded samples, but was not seen in temporarily ischemic samples. Myocyte edema, though common, rarely caused complete capillary occlusions.

Precapillary sphincters and metarterioles in all four groups were only mildly affected by ischemia and reperfusion. Their endothelial and smooth muscle cells contained mitochondria that were extremely vacuolated. Neither of these vessels was ever observed in a constricted, occluded state. Vasoconstriction of arterioles and precapillary sphincters has been suggested as an alternative cause of reflow inhibition in brain (37,48) and heart (45,68). It is possible that the transient inhibition of reflow observed in some of our samples is the result of vasoconstriction. Though none of these vessels were ever observed in a constricted state, the total number actually seen was small. Constriction of a single precapillary sphincter or arteriole would affect flow through a number of distal vessels. Therefore, not finding constricted vessels is difficult to interpret. If vasoconstriction began only at the time reflow was initiated and had ended

prior to the end of the experiment, then it is not surprising that these vessels were not found constricted. Vasoconstriction lasted less than 30 minutes, since reflow had occurred in all tissue at this time. Therefore, the absence of constricted vessels in these samples was not unexpected.

Increased vascular permeability, evidenced by Evans blue tissue staining, was more common in severely ischemic tissues as the period of ischemia was prolonged. Evans blue dye is almost completely bound to plasma proteins, primarily albumin, when given intravenously at low concentrations (1,90). This dye has previously been used to evaluate endothelial permeability (8,9,23,75). In the present study, tissue staining was most severe following 120 minutes of severe ischemia and reflow. Tissue staining paralleled the occurrence of gap formation between endothelial cells lining capillaries and venules. Widening of endothelial cell junctions, gap formation, was most prominent following 120 minutes of ischemia. Evans blue bound to albumin probably escaped into the extravascular space through these gaps.

Increased vascular permeability in these tissues was not the cause of the transient inhibition to reflow which was sometimes seen. Reflow in some well stained tissues was not inhibited. On the other hand, reflow in other samples was inhibited even though they remained unstained. Furthermore, reflow inhibition in severely ischemic tissues peaked after 60 minutes of ischemia, while vascular permeability was not significantly affected until after 120 minutes of ischemia. Increased vascular permeability and reflow inhibition are, therefore, two separate phenomena.

Summary

Coronary microcirculatory vessels in the dog have been identified as being histologically and ultrastructurally very similar to these vessels in man. However, metarterioles, capillaries and venules in the dog are not lined by

"sponge-like" endothelial cells. Furthermore, venules in the dog are often closely associated with nerve endings. Thirty minutes of severe ischemia causes reversible injury to myocytes within the subendocardium under the conditions used in this study. Capillaries and venules are lined by focally swollen endothelial cells that also form blebs at this time. Sixty, 90, and 120 minutes of ischemia causes irreversible myocyte damage and more severe vascular damage that is not reversed by 30 minutes of reperfusion. Transient inhibition of reflow occurred primarily after 60 minutes of ischemia in this model. Capillary and venous alterations were not responsible for causing this reflow inhibition. Transient vasoconstruction of arterioles, precapillary sphincters and metarterioles are thought to be responsible for this inhibition. These findings may be of some clinical importance in man. These findings suggest that reopening acutely occluded coronary vessels may not immediately lead to reflow to all subendocardial tissue. Continued reperfusion for at least 30 minutes may however, be successful.

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