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APPLICATIONS OF THE COLORED MICROSPHERE TECHNIQUE IN THE MAMMALIAN CORONARY MICROCIRCULATION

A Thesis Presented to the School of Graduate Studies and Research of the University of Ottawa in Partial Fulfillment of the Requirements for the Degree of

Doctorate of Philosophy in Physiology

February 1994

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ISBN 0-315-93561-8

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

DEDICATION

To my dear loving parents: the full extent of what I owe to you is immeasurable.

and to my darling wife Lynn, who makes everything worthwhile.

ACKNOWLEDGMENTS

I must first and foremost record my profound appreciation to Dr. Karel Rakusan, for inspirational and scholarly guidance, as well as his unwavering encouragement, friendship, and moral support throughout my course of study. I am genuinely honored to have had an opportunity to work with, and learn from this exceptional scientist. I further owe a great deal to Dr. H. Fred Downey who gave generously of his time and provided me the considerable benefit of his authority in coronary physiology. I also wish to acknowledge Mrs. Ching Kuo and Mrs. Barbara Hebert who so willingly offered their expert technical assistance. Special thanks are also extended to other professors (notably the members of my thesis advisory committee, Dr. George Biro and Dr. David Parry) and graduate students in the department of Physiology, who provided a fertile and friendly environment for scientific discussion and interaction. In particular, I wish to thank my dear friend Dr. Sanjay Batra, whose enthusiasm and creativity were a constant stimulus.

Last, but definitely not least, to my darling wife Lynn who had to endure my repeated frustrations and setbacks while preparing this thesis. I am ever so grateful for her never-ending love, encouragement, and patience along what must occasionally have seemed a road with no end in sight.

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ABSTRACT

The aim of this dissertation was to provide realistic descriptions of two unique properties of the mammalian coronary microcirculation using our novel application of colored microspheres in myocardium.

One important objective was to provide an innovative approach to investigate the controversial issue of whether individual coronary arteries communicate at the microvascular level, thus potentially giving rise to a zone of dual arterial supply. Simultaneous *in vivo* infusion of two distinctly colored microsphere suspensions into the left anterior descending (LAD; red spheres) and left circumflex (LCx; blue spheres) arteries identified a specific interface region of canine myocardium perfused by both arterial branches. Two distinct zones were delineated, and their widths measured. One region was defined as the Interface Transition Zone (ITZ) [5251 ± 770 μm; mean ± SD]. This region was formed by microvessels supplied by the individual parent arteries, and separated tissue containing only one or the other colored microspheres. The second region defined as the Boundary Watershed Zone (BWZ) [3151 ± 611 μm] was formed by capillaries containing sphere aggregates of both colors; it was located exclusively within the ITZ. In addition, the ITZ and BWZ were significantly wider in subepicardial than in subendocardial regions ($p<0.001$).

Our subsequent study investigated the potential lability of the coronary watershed zones. As before, two differently colored microsphere suspensions were infused into the LAD (red spheres) and LCx (blue spheres) arteries of nine dogs. Subsequently, the LAD was ligated and a third set (green) of spheres was

infused into the LCx. Capillaries perfused exclusively by the LAD before occlusion adjacent to the interface contained green microspheres as well as red/green aggregates, indicating lateral extension of the LCx perfusion territory. Results showed that occlusion caused a 24% expansion of the ITZ and a 48% expansion of the BWZ. In addition, all expansions were significantly greater in subepicardial compared to subendocardial regions ($p<0.001$). These results demonstrated the capability of microvascular anastomoses in providing blood flow to the periphery of an ischemic region. Furthermore, the perfusion interface was found to be labile and potentially amenable to manipulation.

We also applied our colored microsphere technique for the characterization of coronary capillary flow direction in rat myocardium. Our initial study determined capillary flow direction in two groups of male Sprague-Dawley rats: 1) pressure overload hypertrophy (n=7) and 2) sham-operated controls (n=10). Chronic pressure overload was induced in neonatal rats by aortic constriction. Six weeks postsurgery, both groups received sequential *in vivo* infusion of two differently colored microsphere suspensions into the left atrial appendage. Histological analysis of 40 μm serial sections revealed that certain coronary capillaries contained microspheres of both colors. A capillary flow vector was established based on the sequence of colors trapped within each aggregate. Examination of flow vectors among neighboring capillaries enabled the characterization of regional capillary flow direction. Results indicated a predominance in concurrent flow, which decreased significantly ($p<0.001$) over a range of capillary rankings. The percentage of concurrent flow was also

significantly lower in subendocardium ($p<0.001$) compared with midmyocardium. Cardiac hypertrophy was not a contributing factor to the above findings. This study provided previously unattainable data regarding transmural capillary flow direction and suggested regional adaptations in coronary microvascular flow.

The suitability of the isolated perfused heart for the characterization of capillary flow direction was subsequently investigated. As a first step, it was necessary to determine overall validity and reliability of this model. This was accomplished by comparison of the data obtained from our *in vivo* study to data obtained using an isolated perfused "Langendorff" set-up for colored microsphere infusions. Of additional interest was the evaluation of capillary flow direction in the younger heart, in light of previous data demonstrating significant alterations in capillary geometry during normal growth.

Capillary flow direction was determined in male rats aged 6-7 weeks (n=10), and 3-weeks (n=34). Comparison of results between our *in vivo* study and the "adult" isolated heart group did not reveal appreciable differences, except in subendocardial regions where slightly higher percentages of concurrent flow were found in the isolated heart group ($p<0.05$). These data confirmed the validity and reliability of the isolated perfused rat heart model for the characterization of capillary flow vectors. Results between the adult and 3-week groups were not significantly different, suggesting that adaptations or changes in capillary flow direction subsequent to the period of post-weaning development were not important.

To consider the effect of acute hypoxia on regional capillary flow direction

in the isolated heart we employed our colored microsphere technique in two series of experiments. In Series A, a control group (n=10) received three infusions of colored microspheres in sequence during normoxic perfusion. An "hypoxic" group (n=12) received the initial two infusions under identical conditions, with the third infusion under conditions of experimental hypoxia. Hearts in Series B (n=5) received two differently colored infusions, the initial under normoxic conditions, and the second infusion during hypoxia. Histologic examination revealed a lower percentage of concurrent flow subsequent to the hypoxic intervention than during the earlier normoxic period, as well as in comparison with normally perfused controls ($p<0.01$). Flow vector observations from Series B depicted lower proportions of concurrent flow compared with the 2-color normoxic interval in "hypoxic" hearts from Series A ($p<0.05$). Reversals in capillary flow direction, suggested by the reorientation of final injected microspheres relative to the positions of earlier injected microspheres, were interpreted as a response to sustain myocyte oxygenation due to severe reductions in arterial oxygen tension.

FOREWARD

Despite the multiplicity of scientific inquiries over the years in the field of coronary microcirculation research, many questions remain unanswered regarding its ultimate structure and function. The present dissertation attempts to provide realistic quantitative data to delineate two distinctive properties of the coronary microcirculation. More specifically:

1) a series of investigations was designed to characterize regional capillary flow direction using a novel technique of non-radioactive colored microsphere infusions in myocardium. For these studies, the rat model provided a logical continuation of previous work in our laboratory in studying the capillary bed under conditions of both normal and pathological growth.

2) this technique was applied in a different manner for experiments aimed at determining sources of microvascular blood flow along the interface of coronary perfusion territories. These experiments were performed in collaboration with Dr. H. Fred Downey from the Department of Physiology, University of North Texas Health Science Center at Fort Worth. In this case, the dog was selected as the appropriate animal model primarily because previous information forming the basis of these studies had been acquired from this species, and because the dog was large enough to permit the surgical instrumentation and monitoring techniques required for this work.

The foregoing description highlights two discrete areas of investigation bearing no definitive relationship apart from the use of colored microspheres as a microvascular labeling technique. This is further reflected in the organizational

style of the dissertation. Therefore, following a general introduction to microsphere techniques, the dissertation is divided into two main parts. Within each part is included a separate introduction as well as individual chapter headings.

Various portions of this dissertation are in the process of, or have recently been published. The following references list the present status of the material presented in this dissertation.

Intercoronary microvascular anastomoses: Chapter 1
CICUTTI, N., RAKUSAN, K., and DOWNEY, H.F. Colored microspheres reveal interarterial microvascular anastomoses in canine myocardium. *Basic Res. Cardiol.* 87: 400-409, 1992

Advancement of myocardial watershed zones: Chapter 2
CICUTTI, N., RAKUSAN, K., and DOWNEY, H.F. Coronary artery occlusion extends perfusion territory boundaries through microvascular collaterals. 1993. *Basic Res. Cardiol.* (Submitted)

Regional capillary flow vectors in myocardium: Chapter 3
CICUTTI, N., and RAKUSAN, K. Microvascular flow vectors in normal and hypertrophic myocardium as determined by the method of colored microspheres. *Microvasc. Res.* 43: 267-275, 1992.

Capillary flow vectors in the isolated perfused heart: Chapter 4
CICUTTI, N., and RAKUSAN, K. Capillary flow direction in the isolated perfused rat heart. *Adv. Exp. Med. Biol. (Oxygen Transport to Tissue XV)* pp 243-251

Capillary flow vectors in the hypoxic heart : Chapter 5
CICUTTI, N., and RAKUSAN, K. The effect of hypoxia on capillary flow direction in the isolated perfused rat heart. *Can. J. Cardiol.* (in press)

GENERAL INTRODUCTION TO MICROSPHERE TECHNIQUES:

In 1967, radioactively labeled microspheres were introduced by Rudolph and Heymann for the measurement of regional blood flow as a percentage of cardiac output. The arterial reference sample refinement was proposed one year later by Makowski et al. (1968) for the precise quantification of regional blood flow. Since that time it has become the standard and preferred method for the measurement of regional myocardial blood flow in both large and small animal models, and in a variety of experimental settings. Most commonly used are insoluble, isotopically labeled, carbonized plastic microspheres having a relative density of 1.30. Since intravascularly injected microspheres are rigid and typically larger than red blood cells, they become trapped in the terminal vascular bed when they meet their limiting vascular diameters. The distribution pattern of microspheres has also been found to correlate closely with actual blood flow distributions (Heymann et al., 1977). However, the precision of the microsphere method largely depends on the number of microspheres localized in the organs as well as in the reference sample (Buckberg et al., 1971), and not on the radioactivity as was once believed.

Accurate determination of myocardial blood flow distribution also requires that microspheres be completely mixed with the blood before entering the coronary circulation. Wicker and Tarazi (1982a, 1982b) showed that microspheres injected into the left ventricle were inadequately mixed, based on their finding that coronary blood flow determinations in rats were significantly less variable with left atrial injection compared with left ventricular injection.

Previous studies have suggested that microsphere aggregation may be a significant problem, with the potential to alter estimates of myocardial blood flow and its transmural distribution based on measurements of tissue radioactivity following injection of radioactive microspheres (Buckberg et al., 1971; Archie et al., 1973; Heymann et al., 1977; Flaim et al.,1984). With this in mind, Korpan (1987) and Korpan and Rakusan (1987) developed a morphometric method using non-radioactive microspheres for measuring regional myocardial blood flow with a high degree of spatial resolution in anesthetized rats. To investigate this problem, they evaluated the effect of injection site (left ventricle vs left atrium), the number of microspheres injected (5×10^6 vs 1.5×10^5), and the effect of left ventricular hypertrophy (induced through chronic pressure overload) on microsphere aggregation patterns in the coronary vasculature of the rat left ventricle. Their results revealed a significantly higher degree of microsphere aggregation, particularly in the subendocardial layers following ventricular injection in comparison with injection via the left atrial appendage. The degree of aggregation was also found to be significantly higher with injection of the larger microsphere dose. In addition, the higher degree of aggregation observed in hypertrophic hearts compared with their sham-operated counterparts was explained by a higher resting coronary blood supply that was more pronounced in the subendocardium due to extended axial streaming expected with ventricular wall thickening.

In agreement with the results of Wicker and Tarazi (1982a, 1982b), but not with the work of Flaim et al. (1984), left atrial injection was preferred over left ventricular injection for more accurate myocardial blood flow determinations. Furthermore, their results indicated that the degree of microsphere aggregation and transmural distribution of aggregates was an important consideration when interpreting data based on measurements of tissue radioactivity following injection of radioactive microspheres.

Overall, however, when correctly applied, the radioactive microsphere method can be relatively simple, accurate, and reliable. The basic requirements that must be fulfilled, as well as potential pitfalls of the method have been extensively discussed (see Korpan,1987). These include:

1. Microspheres must be uniformly distributed in the suspension medium prior to intravascular injection.
2. Immediately following injection, microspheres must be well mixed with and evenly distributed in the circulation such that the concentration of microspheres reaching all arterial branch points is equal.
3. Each sample (organ, tissue specimen, or arterial reference sample) must contain adequate numbers of microspheres to assure acceptable errors and confidence limits.
4. The number of microspheres injected must be accurately determined.
5. Microspheres must be effectively trapped during their first passage through the microcirculation such that that recirculation is insignificant.
6. Microspheres must remain trapped until counted.

7. Microsphere injection, embolization, and reference sample withdrawal should produce insignificant hemodynamic effects.

8. The microsphere method must be reproducible and correlate well with other techniques.

(A thorough discussion of these points is beyond the scope of the present review: for detailed discussion of these, and other factors, the reader is urged to consult the following references: e.g., Buckberg et al., 1971; Archie et al., 1973; Heymann et al., 1977; Flaim et al., 1984; Korpan, 1987).

To avoid some of the potential limitations inherent in the radioactive microsphere technique, alternative approaches utilizing microspheres have been devised. In 1985, a small Los Angeles, California-based company, S.R.P. now known as EZ-TRAC, introduced the use of non-radioactive colored microspheres for the analysis of regional blood flow. This technique is based on a series of steps: acquisition of tissue samples, enzymatic digestion, microsphere collection via centrifugation and finally, concentration analysis with a hemocytometer. Results of this method, as carried out by Shell et al. (1985), indicated that it compared favorably with those obtained using radioactive microspheres. Shortly thereafter, Hale et al. (1988) also successfully employed non-radioactive colored microspheres in a similar fashion for the measurement of regional myocardial blood flow. More recently, an alternative non-radioactive method for measurement of regional myocardial blood flow was developed by Morita et al. (1990) using x-ray fluorescence excitation of microspheres loaded with elements of high atomic number.

Utilizing polystyrene microspheres incorporating dyes with any of five distinct colors, Kowallik et al. (1991) also measured regional myocardial blood flow. In their approach, microspheres were extracted from the heart and blood by digestion with potassium hydroxide and subsequent microfiltration. The dyes were then recovered from the colored microspheres within a defined volume of solvent, and their concentrations were determined by spectrophotometry. It was determined that the separation of composite absorbance spectra by spectrophotometry with the colored microsphere technique was as reliable as the separation of energy spectra by a gamma-counter using the radioactive microsphere technique.

However, unlike radioactive or colored microspheres which have been used strictly for blood flow analysis, it was our belief that colored microspheres could also be used in a number of innovative ways to clarify certain unresolved and contentious issues in the field of coronary microcirculation research. This belief found its roots in the series of investigations presented in this dissertation, to which the colored microsphere technique has been uniquely suited. For example, our colored microsphere technique can be utilized for both *in vivo* and *in vitro* characterization of coronary capillary flow direction. Since the microspheres remain trapped in the terminal vascular bed, a permanent record of microvascular flow activity during the periods of infusion is provided. Histologic examination of the sequence of colored microsphere orientation patterns permits quantification of capillary flow directions.

Additionally, this technique offers the distinct advantage of being applicable to investigations of sources of microvascular blood flow along the junction of perfusion fields in the in situ, working canine heart (through simultaneous infusions of differently colored microspheres into adjacent coronary arteries). In contrast, previous approaches to this problem have required in vitro perfusion of coronary vessels and immediate arrest of the circulation to localize the distribution of perfusion markers (eg. Factor et al., 1978; Przyklenk et al., 1986). Furthermore, as this dissertation will illustrate, our technique allows for the investigation of interventions that can potentially alter the localization of the coronary perfusion interface by infusing additional, colored microspheres after labeling these zones under controlled conditions.

PART A: MICROVASCULAR ANASTOMOSES IN THE CORONARY CIRCULATION

INTRODUCTION

NOTE:

For the purposes of this dissertation the terms "collateral" and "anastomosis" have been applied as synonyms. In the past, the term collateral has occasionally been synonymous with vascular ramification. For example, Schoenmackers (1948) defined collateral vessels as those which connect branches of the same artery (homocoronary anastomoses), and "true" anastomotic vessels as those which connected branches of two separate arteries. Nevertheless, in surveying the available literature, it is evident that to date no general consensus has been reached regarding this terminology.

Therefore, in order to avoid potential confusion, distinctions between intercoronary and intracoronary anastomoses or collaterals in the dissertation are indicated wherever appropriate.

HISTORICAL OVERVIEW

Anatomical Studies

Richard Lower is generally acknowledged as having provided the first depiction of coronary anastomoses in the human heart. His important treatise, *Tractatus de Corde*, published in 1669 described how he visualized fluid injected into one coronary artery pass into another. In discussing the course of the coronary anatomy on the myocardial surface, he described communications by "anastomoses" from vessels originating from opposite regions of the heart. The importance of his findings, however, was not appreciated by either him or his colleagues, due, primarily to a poor awareness at the time regarding the syndrome of coronary heart disease.

The copious nature and complexity of the coronary microcirculation began to be appreciated from the pioneering work of Meigs, when in 1899 he described the richness of the terminal vascular bed from his dye injection experiments of human hearts at autopsy. By 1907, Spalteholtz described an innovative approach to the study of the coronary circulation that permitted him to visualize small arterioles beyond the resolution capacity of the radiographic equipment then available. After surface preparation, he injected both coronary arteries with chrome-yellow gelatin. Upon fixing the heart in formaldehyde it was dehydrated in a series of organic solvents including alcohol and benzene. The heart was then immersed in oil of wintergreen which penetrated the tissue, and rendered the myocardium transparent: the opaque, injected vessels standing out prominently from the cleared parenchyma. On the basis of these early studies as well as continuing investiga-

tions published in his classical text in 1924, Spalteholtz emphatically concluded that the coronary arteries were not end-arteries. His assertions were founded on what he observed as rich anastomoses of all sizes and at all levels connecting the vascular beds of adjacent coronary arteries. The technique of X-ray arteriography utilizing stereo resolution which led to Jamin and Merkel's atlas (1907) of postmortem coronary arteriograms, added impact to Spalteholtz' findings. This led Gross in 1921 to the statement that, at least in the dog heart, "anastomoses in the heart are universal and abundant". By the 1930's and early 1940s the pendulum had begun to swing back, when Schlesinger (1938) denied the existence of anastomoses in the normal human heart, although their techniques were much inferior to methods used by earlier investigators. However, the potential functional importance of these vessels in the heart was emphasized by Blumgart and coworkers (1940), who demonstrated the presence of multiple occlusive lesions in hearts without any evidence of myocardial necrosis and scarring.

Fulton and Thomas (1965) ultimately set the record straight with regard to anastomoses in both normal and diseased human hearts, when they unmistakably described extensive anastomoses with great clarity and precision. Thus, after many years of debate and controversy, owing largely to an immense multiplicity of techniques with their inherent technical and methodological shortcomings, performed on a variety of mammalian species, including man, it had finally become established that large to medium size interarterial collaterals were present in certain mammalian hearts including man, especially in pathologic hearts with coronary obstructive disease. Since such vessels could readily be observed, both

visually and angiographically, models of the coronary collateral circulation typically described macrovascular, interarterial anastomoses originating and terminating proximal to the microcirculation (Fam and McGregor, 1964; Wyatt et al., 1982; Harrison et al., 1986). Experiments designed to estimate the pressure at the origin of collateral vessels had lent physiological support to these models (Wyatt et al., 1982; Harrison et al., 1986).

In addition, numerous anatomical investigations have also described microvascular anastomotic pathways in the human and canine coronary circulations. These investigations have consistently demonstrated abundant anastomoses at both the arteriolar and capillary levels (eg. Brown, 1965; Fulton and Thomas, 1965; Baroldi and Scomazzoni, 1967; Bassinthwaighte et al. 1974; Grayson et al., 1974). The preponderance of microvascular anastomoses has been identified as intracoronary and thus connecting branches of the same parent artery, but intercoronary microvascular anastomoses connecting perfusion territories supplied by different parent coronary arteries have also been proposed to exist. However, Grayson et al. (1974) pointed to the extreme difficulty of visualizing and identifying intercoronary microvascular anastomoses, particularly since the perfusion territory interface comprises a small portion of the vascular system and also because of the considerable uncertainty of delineating the parent or source arteries of microvascular anastomoses.

The collateral circulation of the heart has traditionally been regarded as a potential alternative to a major vascular conduit that has become non-functional from, for example, obstruction of a major coronary artery branch. Thus, a

collateral channel is a rarely used pathway that may be recruited after failure of the original vessel in an effort to sustain normal coronary flow. Early attempts to limit myocardial infarct size were predicated on the premise that tissue deprived of its normal blood supply by coronary occlusion was not uniformly ischemic. Whereas cells in the center of the ischemic region were not expected to survive prolonged coronary artery occlusion, cells near the border of the ischemic region appeared to receive more collateral blood flow (Becker et al., 1973; Bishop et al., 1976; Rivas et al., 1976) and, thus, were in a better position to survive the ischemic insult. Survival of some of these underperfused, but viable cells would explain observations of infarcts smaller than the perfusion territory of the obstructed artery (Schaper 1979; Becker et al., 1983; Bishop et al., 1976). Investigators reasoned that if appropriate protective therapy were instituted, the number of surviving cells could be significantly increased and infarct size reduced (Maroko et al., 1971; Maroko and Braunwald, 1973; Braunwald and Maroko, 1974).

The time course of ischemic cell death has been demonstrated most clearly by studies in canine myocardium where various periods of temporary occlusion have been followed by reperfusion (Reimer and Jennings, 1979). In anesthetized, open-chested dogs, even the most severely ischemic myocytes remained viable for at least 15 min. If reperfusion was established during this time interval, infarction could be prevented. Beyond 15 min of coronary occlusion in this experimental model, increasing numbers of ischemic myocytes became irreversibly injured. By 40 min, much of the subendocardial zone, if severely ischemic, became irrevers-

ibly injured (Reimer and Jennings, 1979). The reason for the transmural wave front of myocardial cell death after coronary occlusion is often explained on the basis of a transmural gradient of collateral blood flow, i.e., that the subendocardial region dies quickly because it is severely ischemic and that the subepicardial region dies more slowly since it is often only moderately ischemic. However, there is evidence that transmural differences in collateral blood flow are not the sole explanation for the transmural wavefront of cell death. In studies in which myocardium has been made totally ischemic *in vivo* or *in vitro*, ultrastructural and metabolic features of cell injury have occured more quickly in the subendocardial region than in the subepicardial region despite the transmural absence of blood flow (Dunn and Griggs,1975; Lowe et al.,1983). Thus, transmural differences in myocardial work load or basal metabolic needs may also contribute to transmural progression of injury.

Beginning in the 1960s, much controversy arose over the existence and width of ischemic gradients at the lateral boundaries of an ischemic region. Observations of intermediate values for biochemical markers of ischemia, tissue oxygen tension, collateral blood flow, and tissue damage in the peripheral portions of the ischemic region supported the view that the ischemic insult was not uniform throughout the collateral-dependent region (Cox et al., 1968; Becker et al., 1973; Bishop et al., 1976; Harken et al., 1978; Vokonas et al., 1978; Hearse and Yellon, 1981; Sladek et al., 1984). Such intermediate values were furthermore accepted as evidence of a lateral "borderzone" of potentially salvageable myocardium around

the center of the ischemic core (Maroko et al., 1971; Becker et al., 1973; Maroko and Braunwald,1973; Braunwald and Maroko, 1974; Sladek et al., 1984).

Nevertheless, the existence of this zone of intermediate perfusion at the lateral boundary of acutely ischemic myocardium concept has been disputed (for a review, see Hearse and Yellon, 1981). Precise techniques to measure blood flow, metabolites, and ischemic injury in tissue near the interface between ischemic and normally perfused regions have produced data showing sharp gradients at the interface (Cohen, 1978; Harken et al.,1978; Murdock et al., 1983; Hearse and Yellon, 1981; Axford-Gatley and Wilson, 1988). Thus earlier findings of intermediate values of ischemic indices may be regarded as artifactual due to the resolving power of the sampling techniques employed which may have resulted in the analysis of specimens which contained tissue from both sides of the is chemic/normal interface (Hearse and Yellon, 1981).

Previous anatomical evidence has suggested that microscopic anastomotic pathways may contribute significantly to coronary collateral blood flow capable of producing a gradient of flow along the border of an acutely ischemic region (Brown, 1965; Fulton and Thomas,1965; Baroldi and Scomazzoni, 1967; Grayson et al., 1974).

A series of studies were designed specifically to identify the existence of such anastomoses in normal hearts at the perfusion territory interface, and in hearts with acute coronary occlusion, (Factor et al., 1978, 1981, 1982; Okun et al., 1979). These investigators injected adjacent coronary perfusion territories of isolated hearts post mortem with differently colored silicone elastomers (Microfil).

They found that each color was restricted to its respective territory, and they described the microvascular anatomy at the interface between the coronary perfusion territories as a series of hairpin capillary loops formed by branching capillaries which always doubled back and ran parallel to the parent vessel. Since no vessels were found to contain both colors of elastomer, the investigators concluded that there were no microvascular connections at the interface of coronary perfusion territories. Along the interface an irregular series of peninsulas extended from one territory into the other, an arrangement thought to account for intermediate values of flow and other variables found in tissue at the border of an acutely ischemic region (Hearse et al., 1981). Despite contrary evidence from earlier anatomical investigations, these studies are frequently cited to support the view that coronary arteries are functional end-arteries with no microvascular anastomoses and lack any capability to produce a gradient of perfusion at the border of an acutely ischemic region.

However, conclusions drawn from the Microfil technique must be questioned due to inherent limitations of the technique itself. These studies utilized post mortem preparations where injections of elastomer were conducted 24 hr post occlusion, thus revealing the microcirculation at that point in time only. As well, the investigators did not demonstrate that intravascular mixing of the colored microfil would have occurred if interarterial connections had existed. The viscosity of the elastomers used by Factor and coworkers (120-200 cP) was considerably higher than used by Bassinthwaighte et al. (1974) (15-25 cP) who

treated the elastomers with diluent prior to infusion in their investigation of the microvascular anatomy in canine myocardium.

Thus, in the work of Factor and colleagues (1979,1981), the first color of elastomer to reach a vessel may have prevented entry by the other color due to the relatively high viscosity of the elastomers, thereby precluding the possibility of identifying interarterial capillary anastomoses. In addition, volume shrinkage of elastomer during tissue preparation has been found to be significant during treatment with organic solvents to render tissue transparent (Bassinthwaighte et al., 1974). If elastomers of two different colors abutted in the same capillary, volume shrinkage of both elastomers during clearing in organic solvents as used by Factor and coworkers (1979,1981) would be expected to pull apart the boundary of the two elastomers. Since the injected mass in the lumen of the capillaries, and not the endothelial cell walls was visualized by epi-illumination in these studies it is likely that a single capillary containing both colors would have appeared as two blind-ended capillaries of different colors.

In contrast to the absence of intercoronary mixing of differently colored silicone elastomers, Przyklenk et al. (1986) observed microvascular mixing of differently colored, low viscosity dyes infused into the left anterior descending (LAD) and left circumflex (LCx) coronary arteries of isolated canine hearts *in vitro*. Microscopic examination of tissue from the perfusion territory interface revealed that 21-22% of the capillaries contained both color dyes, and this proportion of dually labeled capillaries was not altered by postmortem cautery of epicardial collateral vessels. Furthermore there was a positive correlation between

the number of capillary anastomoses and blood flow measured in this region by in vivo administration of radioactive microspheres in the outer edge of the LAD bed after occlusion of the LAD. Not only did this study demonstrate the presence of microvascular anastomoses between adjacent coronary perfusion territories, it suggested that these anastomoses might be functionally important in delivering flow to acutely ischemic myocardium near the interface.

Functional Studies:

Other techniques have been devised in order to address the existence and potential importance of microvascular collateral pathways in the coronary circulation. Evidence from the work of Downey et al. (1974,1981) that collateral flow continued antegradely into the collateral-dependent region, even when the obstructed coronary artery was vented to atmospheric pressure (retrograde flow maneuver) suggested that a portion of the collateral flow reached the collateral-dependent region distal to significant arteriolar resistance. Further indirect evidence of microvascular anastomoses was found when Downey et al. (1987, 1988a, 1988b) and others (Schulz et al., 1973; Diemer at al., 1977; Wichmann et al., 1978) observed that peripheral embolization of the collateral dependent vasculature markedly increased retrograde flow. These findings were explained by retrograde diversion of the microvascular portion of the collateral flow, which had flowed antegradely into the capillaries before embolization.

The results of these studies corroborated earlier findings that clearance of diffusible radioactive indicators exceeded the collateral flow measured retrogradely (Levy et al. 1961; Cibulski et al. 1972). These results are also consistent with a recent study by Scheel et al. (1990) who found evidence of collateral flow attributed to microvascular pathways during retrograde flow diversion following peripheral embolization with microspheres.

Microvascular collaterals were originally postulated by Levy et al. (1961) to explain the differences they observed between retrograde flow and clearance of radioactive rubidium. These channels were not expected to contribute appreciably to retrograde flow, since the resistance to antegrade flow through the extensive capillary bed downstream would be less than that to retrograde flow through the upstream, less dense arteriolar network.

However, alternative explanations for residual antegrade perfusion during retrograde flow diversion are possible. First, perfusion of the ischemic region by vessels originating from the ventricular lumen might provide a source of non-divertible flow if the luminal vasculature does not anastomose at a higher level with the coronary circulation. Such an explanation seems unlikely on the basis of studies which failed to detect significant luminal flow into ischemic myocardium following acute coronary artery obstruction (Fixler et al., 1974; Crystal et al., 1981).

Due to the serpentine nature of the interface between coronary perfusion territories (Factor et al. 1978), precise delineation of these regions on a macroscopic level is a difficult task. If normally perfused tissue is included in sam-

ples of the ischemic region. flow in the normally perfused region would appear as residual collateral flow during retrograde flow diversion. The concept of a microvascular, retrograde resistant component of collateral flow has been disputed by those investigators employing the shadow technique to prevent inadvertent inclusion of normally perfused tissue in samples from the ischemic region.

Hirzel et al. (1976) introduced the "shadow" technique, which allows estimation of the degree of admixture of normal tissue into the ischemic sample. To apply this technique, the control coronary region is perfused with blood containing a radioactive label, while the adjacent experimental region is perfused at equal pressure with non-radioactive blood. Samples of the experimental region are considered contaminated if they contain significant amounts of radioactivity. Application of the shadow technique led Murdock et al. (1983) to conclude that no tissue of intermediate perfusion was present. In other words, sample contamination appeared to account completely for an apparent borderzone of intermediate flow. However, if tissue perfused by microvascular anastomoses along the perfusion territory interface accounts for the residual antegrade flow, this tissue would be identified as containing contaminating overlap flow and therefore be excluded by the shadow technique, since, by definition, the technique would exclude all tissue with a dual vascular supply.

In acutely ischemic myocardium, the residual collateral flow reaching the collateral dependent region during retrograde diversion is comparatively small when expressed as flow per gram of ischemic tissue. On this basis, one might conclude that microvascular anastomoses, if they are responsible for the residual

collateral flow, are functionally unimportant. If indeed microvascular anastomoses are of importance, it is more likely at the periphery of an ischemic region where the greatest benefit would be derived. These distal microvessels, although individually possessing small internal diameters, and thus limited flow, would be effectively compensated by the sheer numerical density of the available microvascular pathways. With more distal sites of coronary artery occlusion, the periphery comprises a greater proportion of the mass of the acutely ischemic region, and studies have shown that the infarcted region, expressed as a percentage of the "region at risk" decreases as the total area at risk becomes smaller (White and Bloor, 1981; Becker et al., 1983; Gumm et al., 1988). Such observations support a significant role for microvascular collaterals protecting small regions of myocardium from ischemic damage.

The results of a recent study by Przyklenk et al. (1992) are also consistent with the notion of microvascular collateral flow to adjacent ischemic tissue during coronary artery occlusion. They investigated the phenomenon of sublethal ischemia followed by reperfusion in dogs undergoing (A) 3 hours of subtotal coronary artery stenosis and 3 hours of reflow, and (B) 3 hours of total occlusion (collateral flow support) followed by reflow. Overall levels of coronary flow were similar in the two preparations as determined through radioactive microsphere techniques. Przyklenk and coworkers hypothesized that the differential effect of stenotic versus collateral perfusion on regional contractile function was probably due to inherent differences in oxygen delivery. In other words, with complete occlusion, collateral flow involving extensive microvascular collateral connections

would likely have provided a greater proportion of blood flow and oxygen delivery to the margins of the occluded bed (ie., lateral borderzone) than elsewhere in the occluded region. In contrast, subtotal stenotic perfusion would result in a comparatively more uniform distribution of blood throughout the reduced flow region.

Sabia et al. (1992) used myocardial contrast echocardiography (MCE) to measure collateral blood flow and determine the functional significance of coronary collaterals in patients with acute myocardial infarction (AMI). Their results strongly supported the presence of a quantitatively significant lateral borderzone within the perfusion bed supplied by collateral flow in a majority of patients with recent AMI. The abundance of collateral flow and the relation between residual flow and infarct size suggested that pre-AMI collaterals rather than post-AMI collaterals played a salutary role in their patients. These recent findings are in agreement with a postmortem study of hearts from patients with AMI by Piek and Becker (1988) who showed that a sizable borderzone existed, and that this zone escaped necrosis during infarct progression.

The technique of peripheral coronary embolization has also been employed to determine what portion of flow identified by the shadow technique was, in fact, attributable to contamination by normally perfused tissue in overlapping perfusion territories. In this approach Yonekura et al. (1987) used the shadow technique to, first, selectively label adjacent coronary perfusion territories with radioactive microspheres. Differently labeled microspheres were injected into the left anterior descending (LAD) and left circumflex (LCx) arteries under identical perfusion

conditions. Subsequently, the perfusion territory of the LAD was massively embolized with 15 μm non-radioactive microspheres to effectively abolish flow in its perfusion field. Microspheres of a third label were then administered into the non-embolized LCx. Dye was then injected into the non-embolized artery so as to partition the perfusion territories macroscopically. Blood flow as reflected by tissue content of each differently labeled microsphere, was determined in embolized tissue near the perfusion boundary that did not contain dye.

In Yonekura's study, tissue samples perfused solely by the embolized LAD would not have been expected to contain microspheres injected into the LCx. Yet, along the perfusion territory boundary, tissue samples consistently showed evidence of perfusion from the LCx, which according to the shadow technique belonged to the perfusion territory of the LCx proper. Thus flow in these boundary samples measured by post-embolization injection of microspheres would not have been expected to change markedly. In fact, flow had decreased significantly in hearts with both indigenous collaterals and particularly so in hearts with well developed collaterals. These results supported the notion that the microcirculation along the perfusion boundaries had access to a dual arterial supply.

What these studies did not address, however, was whether interarterial pressure gradients generated by the selective obstruction of the LAD may have carried embolizing microspheres into the adjacent LCx bed, and thereby reduce flow in tissue perfused by the LCx which might not be part of a borderzone of dual vascular supply.

The foregoing anatomical and physiological investigations provide indirect evidence of flow through microvascular anastomotic vessels even in the absence of coronary occlusion. Although it cannot be entirely ruled out that a portion of this flow might have resulted from higher order collaterals or from temporal variations in arteriolar pressures, a more general interpretation of such observations seems in order.

It is proposed that microvascular anastomoses cross the interface and connect the beds of adjacent source arteries, so that tissue along the interface has a dual arterial supply.

Although we believe that the existence of a lateral borderzone has been rejected or its width underestimated by inappropriate use of the shadow technique, the available data would suggest that the zone is relatively narrow. Could a lateral borderzone only a few millimeters in width be of any practical significance, and furthermore, worthy of investigation?

In the first instance, proof of the existence of a lateral borderzone of dual perfusion would provide new understanding of a naturally occurring mechanism of myocardial protection. While the border region may comprise only a small portion of the tissue rendered ischemic by obstruction of a large coronary artery, this fraction increases rapidly as the site of obstruction becomes more distal. Findings that the percentage of ischemic tissue which infarcts after coronary occlusion decreases as the area of ischemic tissue decreases are in accord with this

concept (Becker et al. 1983; Gumm et al. 1988). Thus, the survival of the borderzone may be of importance following small artery obstruction. Furthermore, a dual arterial supply to microvessels at the border of coronary perfusion territories could provide a compensatory blood supply, thus serving a role in protecting myocardium along the junction of perfusion fields from microemboli entering or forming in the coronary circulation.

STATEMENT OF THE PROBLEM 1

The fundamental question of whether individual coronary arteries communicate at the microvascular level has been the subject of considerable controversy for numerous years. Conceptually, such a pattern of vascular communication could give rise to a zone of dual arterial perfusion. Furthermore, this so called "borderzone" could be functionally important in providing blood flow to ischemic tissue along the coronary perfusion interface in the event of coronary artery occlusion. Nevertheless, there has been a paucity of experimental data supporting this notion. Clearly, more research was required to answer the fundamental question of whether or not coronary perfusion territories are connected by microvascular anastomoses. The inconsistencies of earlier investigations also indicated that a new experimental approach would be required to help resolve this issue. Therefore, given the controversy and conflicting nature of prior work in this field, the principal focus of this portion of the dissertation will be to address the following questions:

1. Do coronary microvessels along the interface of the perfusion territories receive blood flow from separate arterial sources? If this initial question can be answered in the affirmative, then could the morphology of this zone of dual arterial supply be further described quantitatively?

2. If a zone of dual arterial supply along the interface of coronary perfusion territories can be readily identified and accurately measured, would it be

labile or essentially fixed? i.e. could the dimensions and localization of this region be shifted due to the altered hemodynamic conditions prevailing during, for example, coronary artery occlusion? If so, would the coronary perfusion interface become a source of microvascular collateral flow to adjacent, acutely ischemic tissue?

3. Could a detailed morphologic characterization of the coronary perfusion interface, as well as an understanding of mechanisms which may affect it, provide a rational basis for therapeutic interventions aimed at salvaging ischemic myocardium?

CHAPTER 1: COLORED MICROSPHERES REVEAL INTERARTERIAL MICROVASCULAR ANASTOMOSES IN CANINE MYOCARDIUM

INTRODUCTION

As described in the introduction to Part 1 of this dissertation, the mammalian coronary microcirculation is a complex network of anastomosing vessels (eg. Brown, 1965; Fulton,1965; Baroldi and Scomazzoni, 1967; Grayson et al., 1974). While these earlier studies documented the presence of anastomoses between microvessels originating from a common artery, there was little beyond anecdotal evidence supporting the existence of anastomoses between the terminal vascular beds of independent arterial sources. Such communication between coronary perfusion territories could theoretically give rise to a zone of dual arterial perfusion. Furthermore, these anastomoses could be functionally important in providing blood flow to ischemic tissue near the perfusion interface subsequent to acute coronary artery obstruction.

Nevertheless, the borderzone concept has been disputed. Studies utilizing post mortem injection of colored silicone elastomers into neighboring coronary arteries of isolated hearts found no microvascular communications between their respective perfusion territories (Factor et al., 1978, 1981, 1982; Okun et al., 1979). Based upon these observations, the authors stated unequivocally that coronary arteries are true end arteries. In contrast, a study utilizing infusion of low viscosity colored dyes into neighboring coronary arteries of isolated hearts found that

numerous capillaries contained both color dyes along the coronary perfusion interface (Przyklenk et al., 1986).

Clearly, more research is required to answer the fundamental question of whether or not coronary perfusion territories are connected by microvascular anastomoses. The inconsistencies of previous investigations indicated that a new experimental approach would be required to help resolve this issue.

In the present study, a novel technique incorporating simultaneous *in-vivo* infusions of non-radioactive colored microspheres into neighboring coronary arteries of canine hearts was applied to determine if microvessels along the interface of the perfusion territories received blood flow from both arterial sources, and if so, to describe quantitatively the transmural morphology of this zone of dual arterial supply.

METHODS AND MATERIALS

Experimental animal preparation:

Eight adult mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg i.v.), supplemented as needed for stable anesthesia. A left thoracotomy was performed and the pericardium was incised to expose the heart. Ventilation was maintained with a positive pressure respirator. In all dogs, fluid-filled catheters attached to Statham Db23 pressure transducers were positioned in the aorta via a femoral artery for the measurement of mean arterial pressure. In two dogs, a Millar catheter-tip transducer was inserted into the left ventricular chamber

through the left atrial appendage and advanced into the left ventricular lumen. Output from the left ventricular pressure transducer was differentiated electronically to yield left ventricular dP/dt and also used as input to a cardiotachometer. A catheter was inserted into a femoral vein for infusion of additional anesthetic and fluids. Arterial blood was sampled frequently for measuring blood gas composition, and the respirator was adjusted to maintain arterial blood gases within physiological limits.

The left anterior descending (LAD) and left circumflex (LCx) coronary arteries were isolated for approximately 3 cm near their origins. Twenty gauge Angiocath catheters were inserted into each coronary artery, and attached through narrow gauge catheters to syringe pumps for infusion of colored microspheres. In two dogs, LAD and LCx blood flows were measured electromagnetically (Carolina Medical Electronics electromagnetic flowmeter) by transducers placed distal to the Angiocath catheters. Blood pressures and other hemodynamic variables were recorded on a SensorMedics R611 polygraph.

Colored microsphere procedure:

Non-radioactive red and blue microspheres with diameters of 12 ± 1.9 μm (five experiments) and 10 ± 0.23 μm (three experiments) were purchased from E-Z Trac, Los Angeles, CA. Prior to injection, the microspheres were suspended at a concentration of 6 x 10^6/ml in saline with .01% Tween solution and agitated in

a vortex mixer for one minute. Microsphere aggregates were not observed during microscopic examination of the suspension.

Thereafter, two differently colored 5 ml microsphere aliquots, each containing approximately 30 $x10^6$ red or blue microspheres were infused simultaneously into the LAD and LCx, respectively, at constant and identical rates of 0.5 ml/min for 10 minutes (Plate 1). As demonstrated in earlier pilot experiments, this number of microspheres clearly delineated the perfusion territories and labeled a sufficient number of capillaries in the interface region to measure a zone of dual arterial supply.

Tissue sampling procedure:

After infusion of the microspheres was concluded, the animals were euthanized by intravenous administration of potassium chloride, the hearts were excised, and 3 to 5 transmural blocks of left ventricular tissue, approximately 1.5 cm^2 in cross-section, were cut along the interface between the LAD and LCx. These samples were quick frozen for cryotomic preparation of 40 µm serial sections. Morphometric data from eight hearts were compiled and analyzed. Three representative tissue blocks, with 20 sections cut per block (amounting to approximately 8% of an average left-ventricular wall thickness of 10mm), were sampled from each of the eight hearts (see sampling scheme below):

8 (hearts) x 3 (blocks/heart) x 20 (sections/block) = 480 sections

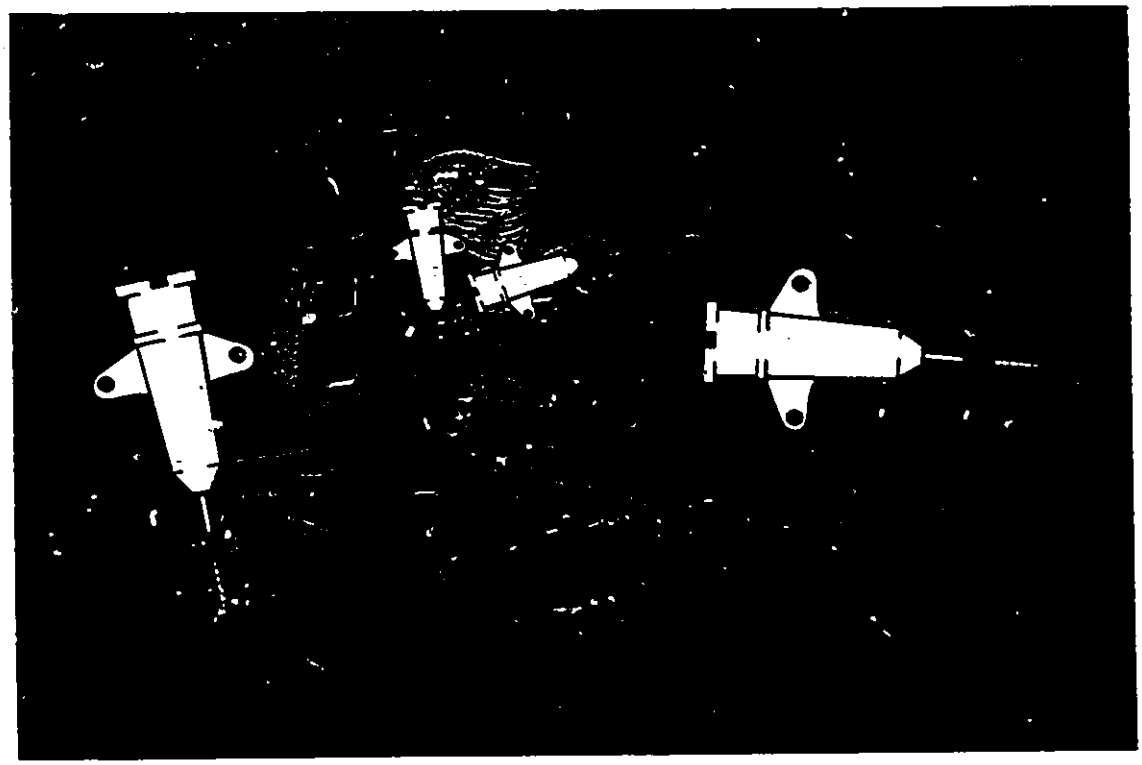


Plate 1 Illustration depicting the placement of catheters for the simultaneous infusion of distinctly colored microsphere suspensions into the left anterior descending (LAD) and left circumflex (LCx) coronary arteries of canine myocardium.

Data derived from the individual tissue blocks within each heart were subsequently pooled. Morphometric analysis was limited to the upper 10 (EPI) and bottom 10 (ENDO) sections of each tissue block for all hearts. Accordingly, each heart yielded a pooled total of 60 sections, 30 (EPI) and 30 (ENDO), for a total of 480 sections incorporating both ITZ and BWZ observations.

Microscopic and Morphometric Analysis:

Scale drawings were constructed of the microsphere distribution within individual tissue sections with the aid of a drawing cylinder attachment linked to an Olympus BH-2 microscope. Particular emphasis was placed on clearly demarcating the distribution of red and blue microspheres in fields where both colors were visible. Detailed microscopy revealed clustering of microspheres in their respective perfusion territories (red microspheres in the LAD region beyond the interface zone and blue microspheres in the LCx region beyond the interface zone). Only rarely was a microsphere of an alternate color observed within a perfusion territory distant from the interface. However, at the interface a region of variable width contained both colors of infused microspheres. Numerous capillaries within this region were found to contain microspheres of both colors. Figure 1 illustrates the distribution of the different colored microspheres observed at the perfusion territory interface.

At the interface, two boundary zones were identified and their widths measured (Fig. 1). The Interface Transition Zone (ITZ) was defined as the region containing both red and blue microspheres. This was the maximum distance

which microspheres supplied by one coronary artery regularly permeated the perfusion territory of the other parent artery. The Boundary Watershed Zone (BWZ) was defined as the region comprising microsphere aggregates of both colors. This zone as measured, depicted the greatest distance between capillaries containing bicolored sphere aggregates. Accordingly, the BWZ was always narrower and located exclusively within the ITZ. The presence of bicolored aggregates entrapped within the same microvessel provided evidence that the region received blood flow from both parent coronary arteries (Plates 2a and 2b).

The spatial positions of microspheres in selected fields along the perfusion interface were subsequently digitized on a graphic tablet linked to an image analysis software program (Bioquant IV, R&M Biometrics, Inc., Nashville, Tn). For each tissue section, parallel line segments were drawn to delineate the outer boundaries of the ITZ and BWZ. The maximum widths of the tv o zones were computed from a perpendicular, midpoint line drawn between the parallel outer boundary markers.

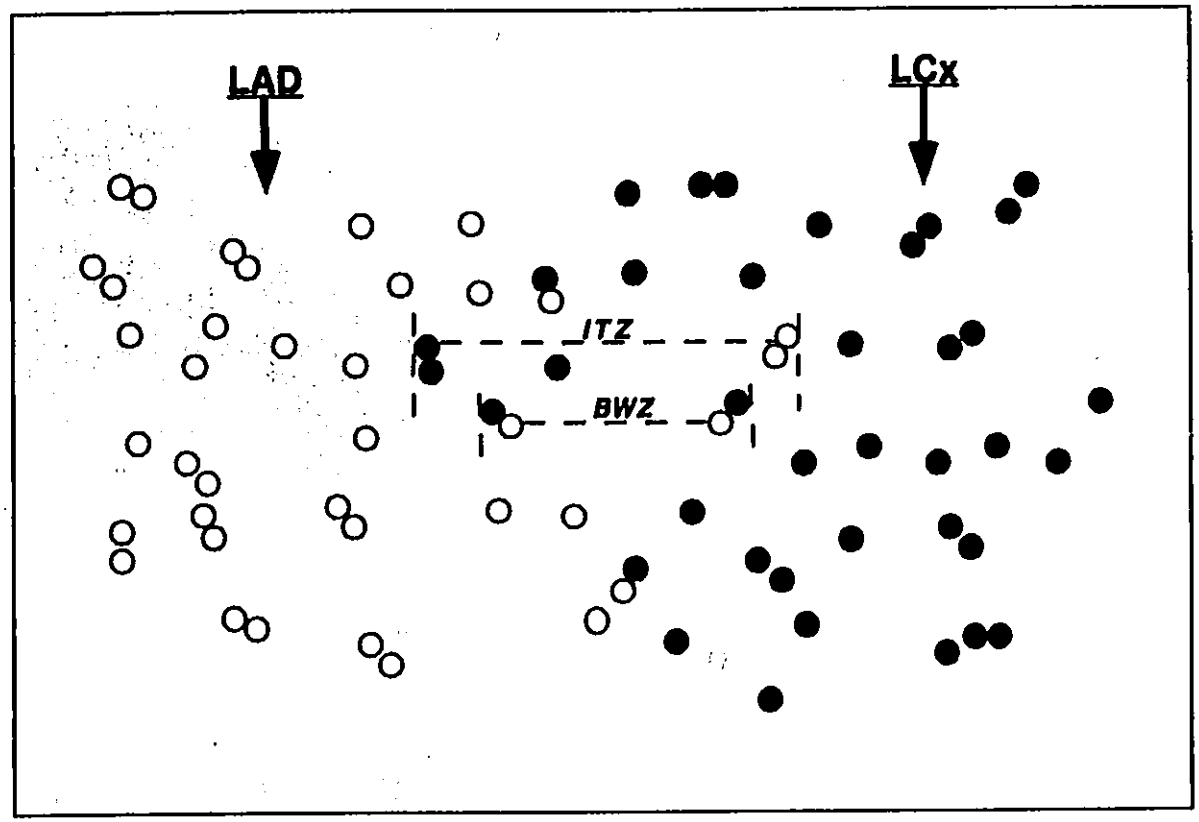


Figure 1. Schematic representation of the distribution of differently colored microspheres, as observed microscopically along the interface of the LAD and LCx perfusion territories. Solid circles represent red microspheres infused into the LCx, and open circles are blue microspheres infused simultaneously into the LAD. Two zones are illustrated: 1) **Interface Transition Zone (ITZ)**, depicting the maximum distance between alternate colored spheres entrapped within the perfusion field of each parent artery. 2) **Boundary Watershed Zone (BWZ)**, depicting the maximum distance between capillaries containing sphere aggregates of both colors. Note absence of the alternate color beyond the ITZ.

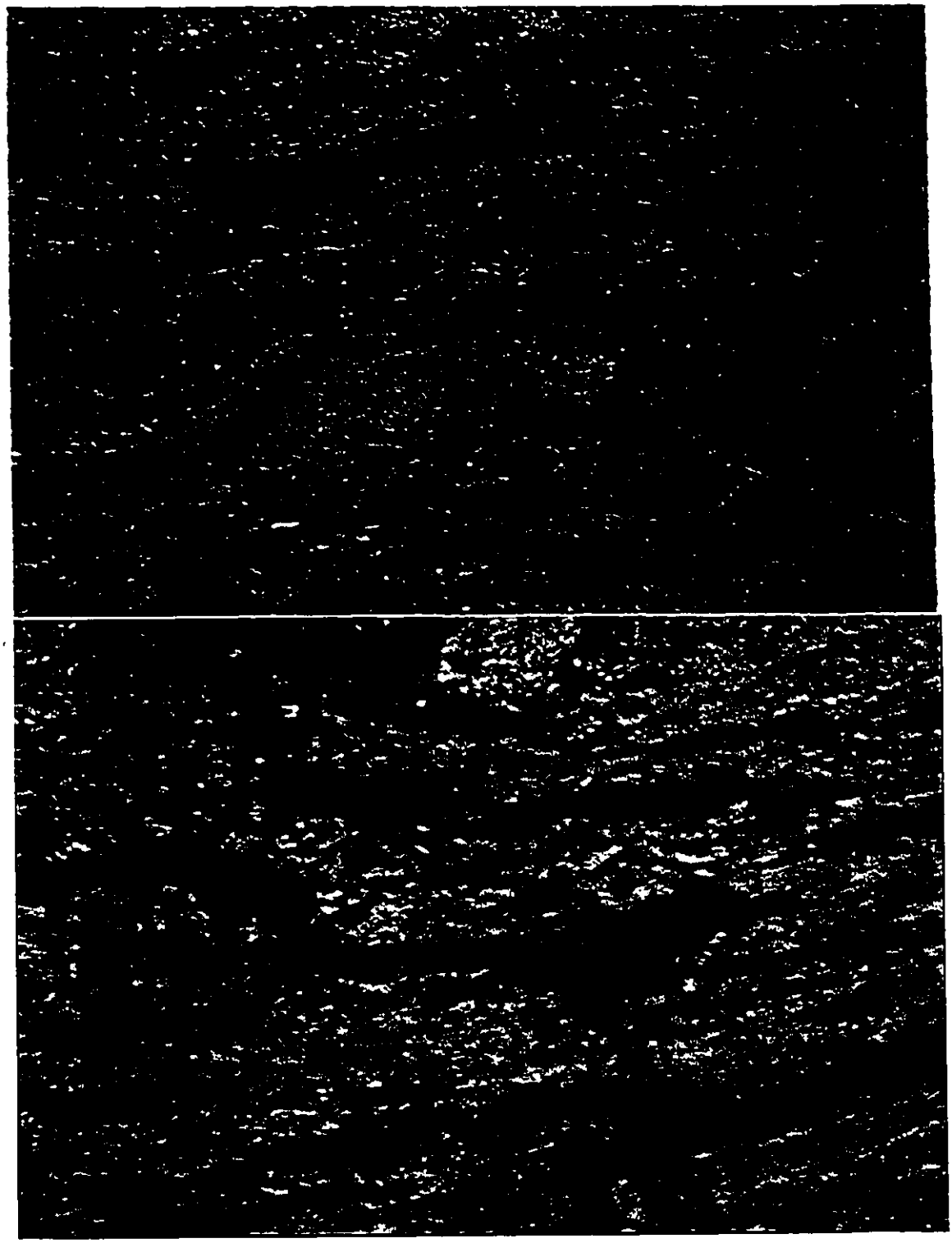


Plate 2 Light photomicrograph of a section of canine myocardium showing aggregates of red and blue colored microspheres within the Boundary Watershed Zone (BWZ), at two magnifications: 250x, and 500x.

RESULTS

Hemodynamic data:

For five experiments utilizing the 12 ± 1.9 μm microspheres, mean arterial blood pressure remained stable (114 ± 8 mmHg; mean ± SD) during the first three minutes of infusion. Mean arterial blood pressure was 86 ± 16 mmHg at five minutes, 75 ± 15 mmHg at eight minutes, and 70 ± 11 mmHg at ten minutes, when the microsphere infusions were terminated.

For three experiments utilizing the 10 ± 0.23 μm microspheres, mean arterial pressure also remained stable (109 ± 6 mmHg; mean ± SD) during the first three minutes of infusion. Mean arterial blood pressure was 110 ± 9 mmHg at five minutes, 102 ± 8 mmHg at eight minutes, and 98 ± 12 mmHg by the ten minute mark, when infusions were concluded. Also monitored in two of these experiments were blood flow in the LAD and LCx during the infusion period, as well as left ventricular pressure and its first derivative, dP/dt. In these experiments, coronary blood flow and other hemodynamic variables were stable throughout and after the 10 minute infusion period. After infusion of microspheres, the coronary arteries dilated normally following a 20-sec coronary occlusion. There was no decrement of left ventricular function in either experiment. A recording of these variables is illustrated in Fig. 2.

Morphometric measurements:

To verify reproducibility in measurement technique, test-retest comparisons were computed for measurements of ITZ and BWZ widths in 30 randomly

selected tissue sections. These test-retest comparisons differed by 5% and 6% respectively, for the ITZ and BWZ zones. In addition, the coefficients of variation of repeated measurements of the same BWZ and ITZ were 2.3% and 3.5% respectively. These values suggested a sufficient degree of reproducibility for the morphometric procedures used in the investigation.

Frequency histograms depicting the distribution of ITZ and BWZ widths are illustrated in Figure 3. The ITZ ranged from 3519 μm to 7613 μm, with the mean ITZ width being 5251 ± 770 μm; mean ± SD. The BWZ ranged from 1745 μm to 4987 μm, with a mean value of 3151 ± 611 μm. The mean width of the ITZ was 5604 ± 772 μm in subepicardial tissue and 4898 ± 586 μm in subendocardial tissue ($p < .001$; 2 factor ANOVA). The mean width of the BWZ was 3437 ± 589 μm in subepicardial tissue and 2873 ± 492 μm in subendocardial tissue ($p < .001$; 2 factor ANOVA). These findings are illustrated in Fig. 4.

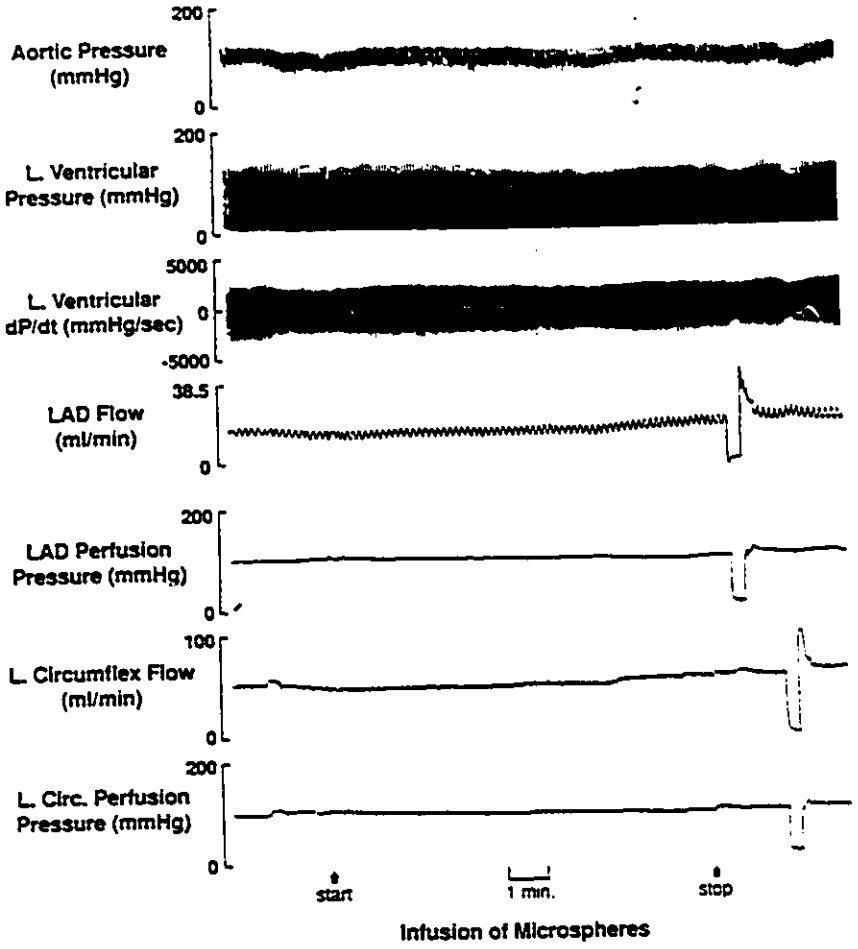


Figure 2. Record of coronary and systemic hemodynamic variables and left ventricular dP/dt during infusions of 30 million 10 μm microspheres into the LAD and LCx coronary arteries. Note stability of left ventricular function, coronary blood flows and perfusion pressures, systemic arterial pressure, and absence of arrythmias during the infusions. Also note normal coronary reactive hyperemic responses to 20-sec periods of ischemia following the infusions.

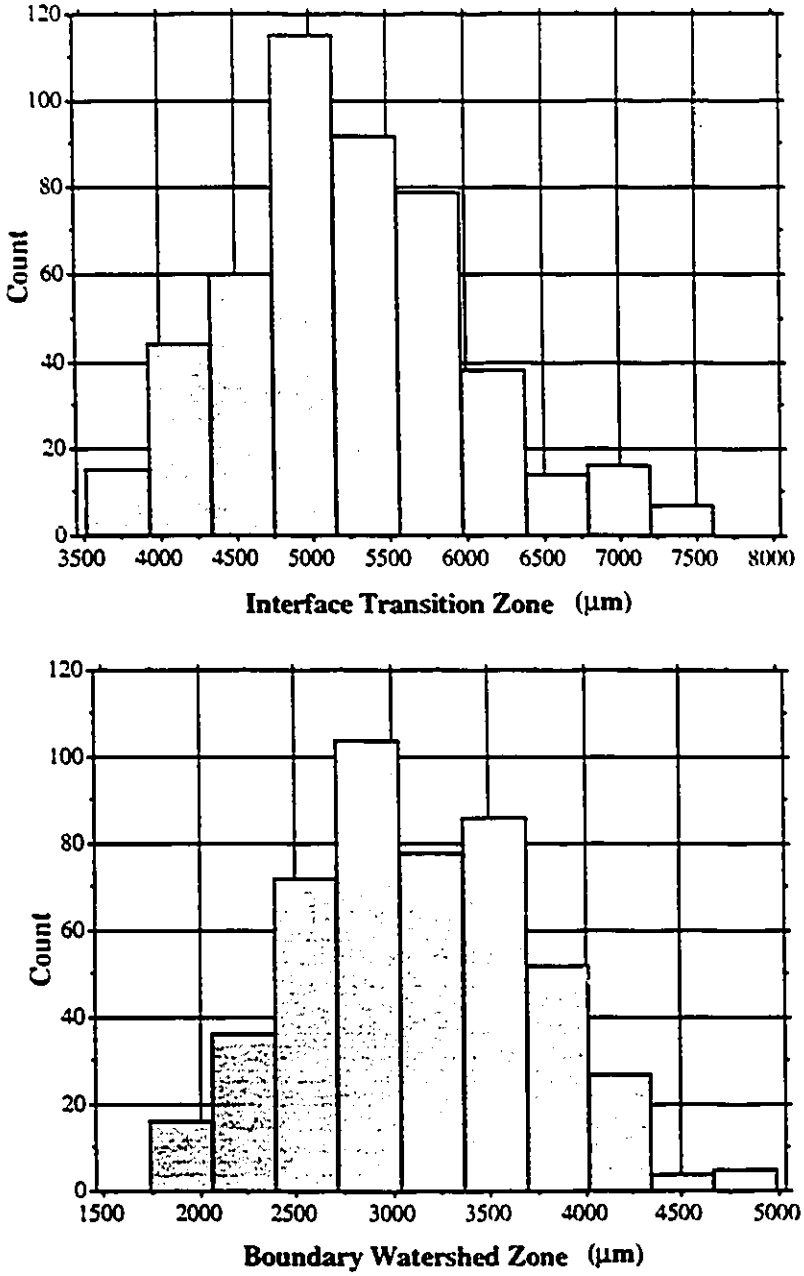


Figure 3. Frequency histograms illustrating the distribution of InterfaceTransition Zone and Boundary Watershed Zone widths in μm. The two distributions combine all measurements obtained from both subepicardial and subendocardial regions of the individual hearts.

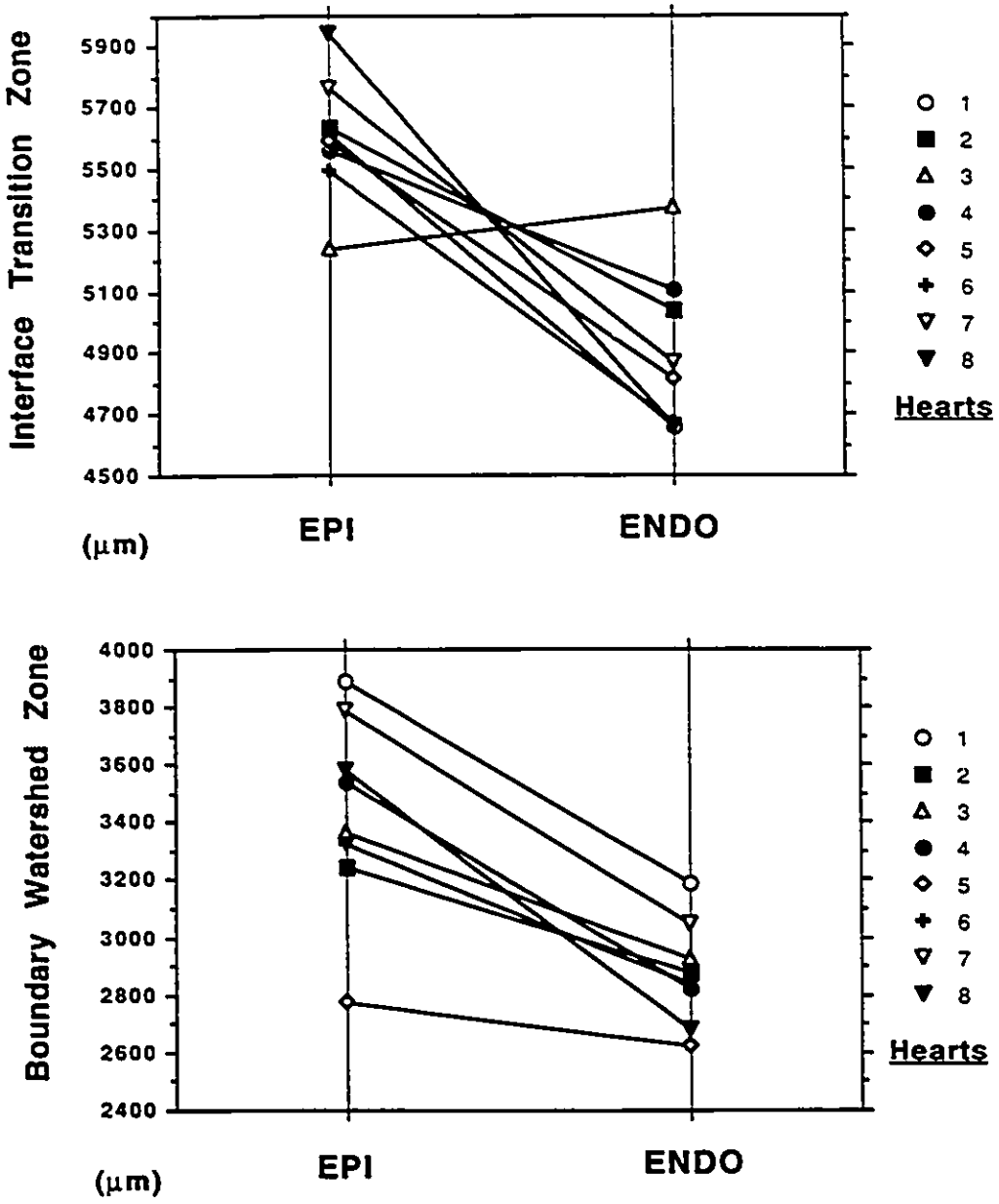


Figure 4. Graphical representation of the effect of regional differences (EPI vs ENDO) on Interface Transition Zone and Boundary Watershed Zone measurements for the individual hearts. Overall, for both zone classifications, measurements of width from subepicardial regions were significantly greater ($p < 0.001$) than in subendocardial regions. These results suggest a transmural narrowing of the coronary perfusion interface.

DISCUSSION

The preeminent finding of this investigation was that myocardial capillaries at the junction of coronary perfusion territories are supplied with blood from both coronary arteries. Since microvascular anastomoses clearly connect coronary perfusion territories, these arteries are not "end arteries." In addition, the width of this zone of dual perfusion was significantly greater in subepicardial tissue than in subendocardial tissue. These findings resulted from application of a new technique to microscopically label adjacent perfusion territories of coronary arteries in the *in situ*, working canine heart.

Previous attempts to delineate the source of arterial supply to tissue at the interface between coronary perfusion fields have yielded controversial and conflicting results. Several studies have reported the absence of a lateral borderzone of intermediate perfusion in hearts with acute coronary occlusion. (eg Okun et al. 1979; Factor et al. 1978,1981,1982) These investigators found no microvascular mixing of differently colored silicone elastomers injected post mortem into the LAD and LCx coronary arteries; rather, they described end-capillary loops at the perfusion territory interface. These studies are frequently cited as "proof" that no lateral borderzone exists in the mammalian coronary circulation.

In contrast, Przyklenk et al. (1986) infused low viscosity, different colored dyes into coronary arteries of isolated hearts and observed numerous capillaries containing both colors in tissue at the interface of their perfusion territories. The low viscosity dyes used by Przyklenk and co-workers may have facilitated mixing

of confluent microvascular streams, whereas adequate mixing might have been impeded by the higher viscosity elastomers employed by Factor and co-workers, thereby precluding the detection of interarterial capillary connections. Przyklenk et al. (1986), however, did not measure the width of the region containing capillaries with both colors of dye. All of these previous studies were performed in isolated, perfused, non-working hearts. In the present study, the regional coronary microcirculation was labeled with different colored microspheres infused under normal, *in situ* working conditions. The presence of both microsphere colors in capillaries across the lateral border of adjacent perfusion territories clearly demonstrates that these microvessels received blood from both parent coronary arteries. Vessels containing such aggregates were frequently observed. Therefore, a zone of dual arterial perfusion could be readily identified, and for the first time, its width accurately measured.

Since it is improbable that the outermost vessels with a dual arterial supply would have been always labeled, the width of the BWZ as determined morphometrically in this investigation, is most likely an underestimate of the width of tissue with a dual arterial supply. Also measured was the width of the ITZ, i.e., tissue supplied by both parent arteries, but not necessarily containing dually labeled capillaries. Therefore, it is reasonable to propose that actual watershed zone dimensions are, in fact, intermediate between the measured ITZ and BWZ, ie., between approximately 3000 and 5000 μm.

Examination of the widths of the BWZ and ITZ across the left ventricular free wall also indicated smaller dimensions of both zones in the subendocardial

regions. It is of interest to note that our investigation of capillary flow vectors in rat myocardium (described in Part 2 of the dissertation) suggests a transmural narrowing and closer spacing among myocardial arteriolar units, as the circumferential dimensions of the heart decrease towards the endocardial surface.

Investigations of a lateral borderzone of intermediate blood flow utilizing the radioactive microsphere technique have also yielded conflicting results (eg. Becker et al., 1973; Murdock et al., 1983). Unfortunately, this technique loses precision in small sections of tissue in the border region, since a low microsphere count in the sample may not allow for an accurate determination of blood flow (Buckberg et al., 1971). In an effort to enhance experimental objectivity, Hirzel et al. (1976) introduced the "shadow" technique, which allows evaluation of the extent of intermixing of normal and ischemic tissue. To apply this technique, the control coronary region is perfused with blood containing a radioactive label, while the adjacent experimental region is perfused at equal pressure with non-radioactive blood. Samples of the experimental region were considered contaminated if they contained significant amounts of radioactivity. Application of the shadow technique to the study of Murdock et al. (1983) led these investigators to conclude that no tissue of intermediate perfusion was present. They concluded that sample contamination was responsible for an apparent borderzone of intermediate flow. However, if we accept the possibility that microvascular anastomoses along the interface have a dual arterial supply, the shadow technique cannot be used to partition flow at the interface, since, by definition the technique would exclude dually perfused tissue.

The zone of microvessels containing microspheres of both colors was limited strictly to the interface region. Thus, this admixture of microspheres within the interface resulted from microvascular anastomoses, and not from higher level coronary collateral connections. Higher level collaterals would have distributed alternative color spheres throughout wide areas of the perfusion territories of both coronary branches, and not simply within the interface. Furthermore, both the LAD and LCx were normally perfused from the aorta, so interarterial pressure gradients and collateral blood flow were minimized.

A large number of microspheres was infused into each coronary artery in order to increase the probability of detecting capillaries with dual arterial perfusion. Notwithstanding, only a minimal number of capillaries with a dual arterial supply were likely to be labeled. Bassingthwaighte et al. (1974) reported 3100-3800 capillaries/mm^2 in canine myocardium with capillary lengths from 500-1000 μm. Taking the minimum capillary density and longest length, this would result in a predicted capillary number of $3.1 \times 10^6/cm^3$. For an LAD perfusion territory comprising 25 grams of tissue, approximately 7.8×10^8 capillaries could potentially be embolized by the infusion of microspheres. If each of 3×10^7 microspheres infused into the LAD obstructed one capillary, this would amount to 4% of the total number of capillaries. Since numerous vessels trapped more than one microsphere, the number of capillaries embolized was no doubt considerably less than 4%.

Although a large number of microspheres was infused into the LAD and LCx, only a small fraction of their respective microvascular beds were embolized. However, one might still question whether arterial pressure or any other hemodynamic parameter is affected by infusing 30 million microspheres into each artery. Hemodynamic measurements indicated a small but statistically significant decrement in mean arterial pressure in those experiments using the larger and less uniform 12 μm diameter spheres. Experiments using the 10 μm spheres found no significant decrement in arterial pressure, left ventricular contractile function, or coronary blood flow. These results warranted the use of the smaller and more uniform diameter microspheres in future investigations employing this technique. On the other hand, morphometric measurements as determined by microsphere distributions were not significantly different in experiments utilizing either the 12 μm or 10 μm microspheres. This uniformity in measurement permitted us to pool the morphometric data derived from both series of experiments.

The functional significance of a relatively narrow region of tissue along the interface of coronary perfusion fields remains to be determined. Tissue protected by such dual perfusion would constitute a relatively small part of tissue jeopardized by a proximal coronary artery obstruction. On the other hand, occlusion of a small artery or arteriole at the interface might be compensated by the dual perfusion sources. Although not so widely recognized, microcirculatory embolization has been implicated as a precipitating factor in patients suffering sudden ischemic cardiac death (Falk 1985; Davies et al., 1986), and in the pathogenesis of cardiomyopathy caused by Trypanosoma Cruzi (Rossi, 1990).

Thus, it seems reasonable to suggest that microvascular anastomoses could play a role in protecting tissue in the BWZ and likely the ITZ as well from local ischemic insult. This is in contrast to the sizable collateral vessels required to protect tissue from large vessel occlusion.

In conclusion, this investigation confirmed the presence of microvascular anastomoses emanating from independent arterial sources. By utilizing our colored microsphere technique, we were able to identify unequivocally a specific interface region of canine myocardium that was perfused by both arterial branches. Systematic measurements across the left ventricular free wall further revealed a significant narrowing from subepicardial to subendocardial regions in this zone of dual arterial supply. This study, therefore, provides previously unattainable knowledge regarding the transmural microvascular morphology along the boundary of coronary perfusion territories. A further investigation designed to examine the lability of the coronary perfusion interface is presented in the following chapter.

CHAPTER 2: CORONARY ARTERY OCCLUSION EXTENDS PERFUSION TERRITORY BOUNDARIES THROUGH MICROVASCULAR COLLATERALS

INTRODUCTION

In the previous chapter we indicated that the essential question of whether independent coronary arteries communicate at the microvascular level has been the subject of considerable controversy for numerous years. We further pointed to the need for an innovative and more discriminating technique which would help resolve this issue.

Our novel methodological approach to this problem, utilizing differently colored microspheres infused separately into adjacent coronary arteries of in situ working canine hearts, indicated that a zone of dual arterial supply along the interface of coronary perfusion territories could be readily identified and accurately measured. Furthermore, capillaries containing both microsphere colors were limited almost exclusively to the perfusion interface, thus challenging on fundamental grounds the notion that coronary arteries are true "end-arteries".

What these results did not fully elucidate, however, was whether the coronary perfusion interface was labile, and therefore potentially responsive to pathophysiological mechanisms or pharmacological intervention. As a first step to address this question, we investigated whether the width and localization of this interface could be shifted by the altered hemodynamic conditions prevailing during acute coronary artery occlusion. We utilized the colored microsphere

technique to determine if the interface tissue, normally perfused by both coronary arteries, was still perfused after one parent artery was obstructed. A further important objective was to determine if this perfusion interface became a source of microvascular collateral flow to adjacent, acutely ischemic myocardium.

METHODS AND MATERIALS

Experimental animal preparation:

Surgical preparation of nine adult mongrel dogs (21.7 ± 3.8 kg; mean ± SD) followed the procedure outlined in Chapter 1. As in the previous experiments, the left anterior descending (LAD) and left circumflex (LCx) coronary arteries were isolated for approximately 2 cm from their origins and dissected free from the surrounding fat and connective tissue. Additionally, a (4/0) silk snare was positioned loosely around the LAD. Twenty gauge Angiocath catheters were inserted into each coronary artery, and attached through narrow gauge catheters to syringe pumps for infusion of colored microspheres. LAD and LCx blood flows were measured electromagnetically (Carolina Medical Electronics electromagnetic flowmeter) by transducers placed distal to the Angiocath catheters. Blood pressures and other hemodynamic variables were recorded on a SensorMedics R611 polygraph. After recording of baseline hemodynamic measurements, the next phase of the experiment involved a complete occlusion of the LAD. This was brought about by abruptly tightening the LAD snare. The occlusion effectively abolished blood flow in the LAD branch. Left ventricular function, systemic

arterial pressure, and coronary blood flow in the LCx artery continued to be monitored for the duration of the experiment.

Colored microsphere procedure:

Initially, two distinctly colored 5 ml microsphere aliquots, each containing approximately 3 x10^7 red or blue microspheres were infused simultaneously into the LAD and LCx, respectively, at constant and identical rates of 0.5 ml/min for 10 minutes. As discussed in Chapter 1, this number of microspheres clearly delineated the perfusion territories and labeled a sufficient number of capillaries in the interface region to measure a zone of dual arterial supply. Subsequently, the LAD was abruptly occluded, and after a five minute period of stabilization, a third 5 ml solution containing 3 x10^7 green microspheres was infused into the patent LCx artery. After infusion of the microspheres was concluded, the animals were euthanized by intravenous administration of potassium chloride, and their hearts were excised.

Tissue sampling and preparation from the nine hearts was conducted in a manner identical to that in the previous study. As before, data obtained from the individual tissue blocks within each heart were pooled. Three representative tissue blocks approximately 1.5 cm^2 in cross-section were cut along the interface between the LAD and LCx. Twenty sections were cut per block from each of the nine hearts (see sampling scheme below).

9 (hearts) x 3 (blocks/heart) x 20 (sections/block) = 540 sections

These blocks were quick frozen for cryotomic preparation of 40 μm serial sections. Morphometric analysis was limited to the upper 10 (EPI) and bottom 10 (ENDO) sections of each tissue block for all hearts. Sampling from these Epi-Endo regions yielded 270 tissue sections for each of the two zones. Each heart thus provided a pooled total of 60 sections. 30 (EPI) and 30 (ENDO); for 540 sections comprising the zone classifications described.

Microscopic and Morphometric Analysis:

Particular emphasis was placed on clearly demarcating the regions of tissue where red, blue and green microspheres were distributed. Detailed microscopic analysis revealed the homogenous distribution of the initial microsphere infusions in their respective perfusion territories (red microspheres in the LAD region beyond the interface zone and blue microspheres in the LCx region beyond the interface zone). Only rarely was a microsphere of an alternate color observed within a perfusion territory distant from the interface. However, at the interface, a zone of variable width was identified which contained both colors of initially infused microspheres. Figure 5 schematically illustrates the distribution of the different colored microspheres as would be observed at the perfusion territory interface.

At the interface, two baseline, boundary zones were identified and their widths measured (Fig. 5). The detailed morphometric methods describing these measurements have previously been described in Chapter 1. Briefly, the Interface Transition Zone (ITZ) was defined as the region containing both red and blue

microspheres. This was the maximum distance that microspheres supplied by one coronary artery regularly permeated the perfusion territory of the other parent artery. The Boundary Watershed Zone (BWZ) was defined as the region comprising microsphere aggregates of both colors. This zone as measured. depicted the greatest distance between capillaries containing bicolored sphere aggregates. The presence of bicolored aggregates entrapped within the same microvessel provided evidence that the region received blood flow from both parent coronary arteries.

Microspheres of the third infusion were also found in the ITZ and BWZ. Prior screening and analysis from thirty randomly selected tissue sections in three hearts from both subepicardial and subendocardial regions indicated that the numbers of green microspheres as well the numbers of green/red aggregates within the baseline BWZ region, were found in comparable numbers to the numbers of blue microspheres and blue/red aggregates (1-Factor ANOVA). These preliminary observations suggested that the coronary perfusion interface was receiving a significant compensatory blood supply subsequent to LAD occlusion.

Furthermore, capillaries in the LAD region adjacent to the interface were found to contain green microspheres as well as green/red aggregates (see Fig. 5 and Plate 3a and 3b). The numerical density of the final color of injected microspheres as a function of distance from the baseline perfusion interface was additionally determined. Upon identification of the baseline ITZ and BWZ boundaries, the numbers of green microspheres found either singly, or as aggregates were recorded within each tissue section at 500 μm intervals up to a

lateral distance of 2500 μm from the ITZ boundary in the perfusion field of the LAD artery. Preliminary microscopic observations of sphere densities from randomly selected tissue sections in each of the individual hearts indicated that critical examination up to a range of approximately 2500 μm from the baseline ITZ boundary was justified. Measurements beyond this distance were not warranted either due to lack of available tissue area in certain cases, or simply due to extremely low microsphere counts. From within the predefined distance intervals described above, the precise locations at which the densities of green microspheres as well as green/red sphere aggregates decreased significantly were compared to the baseline ITZ and BWZ boundaries, respectively. These positions, within individual tissue sections identified the lateral extensions into the occluded region of the baseline ITZ and BWZ boundaries.

The spatial positions of microspheres in selected fields along the perfusion interface were subsequently digitized on a graphic tablet linked to an image analysis software program (Bioquant IV, R&M Biometrics, Inc., Nashville, TN). For each tissue section, parallel line segments were drawn to delineate the outer boundaries of the ITZ and BWZ. The maximum widths of the two zones were computed from a perpendicular, midpoint line drawn between the parallel outer boundary markers. Similarly, the extended perfusion boundaries were computed by drawing perpendicular midpoint lines from the boundary limits of the ITZ and BWZ to their lateral extensions into the LAD region.

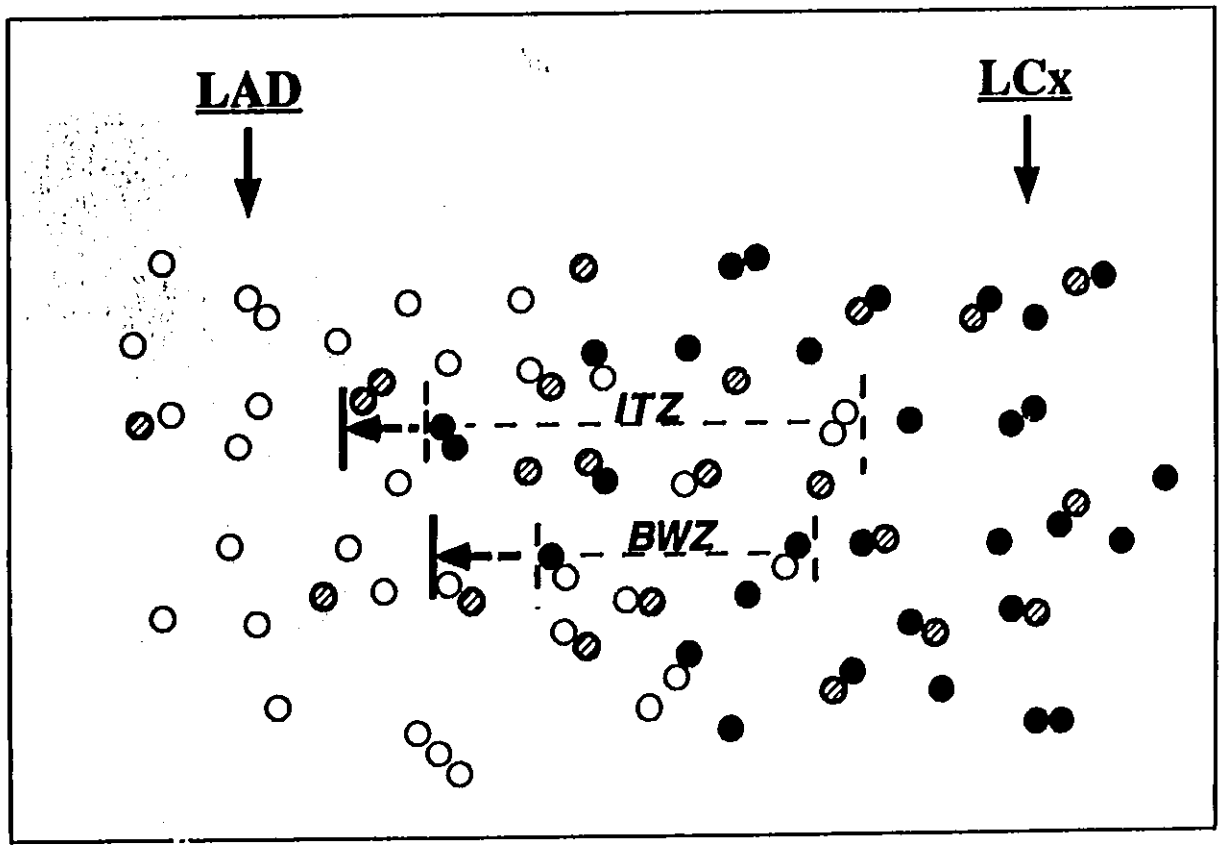
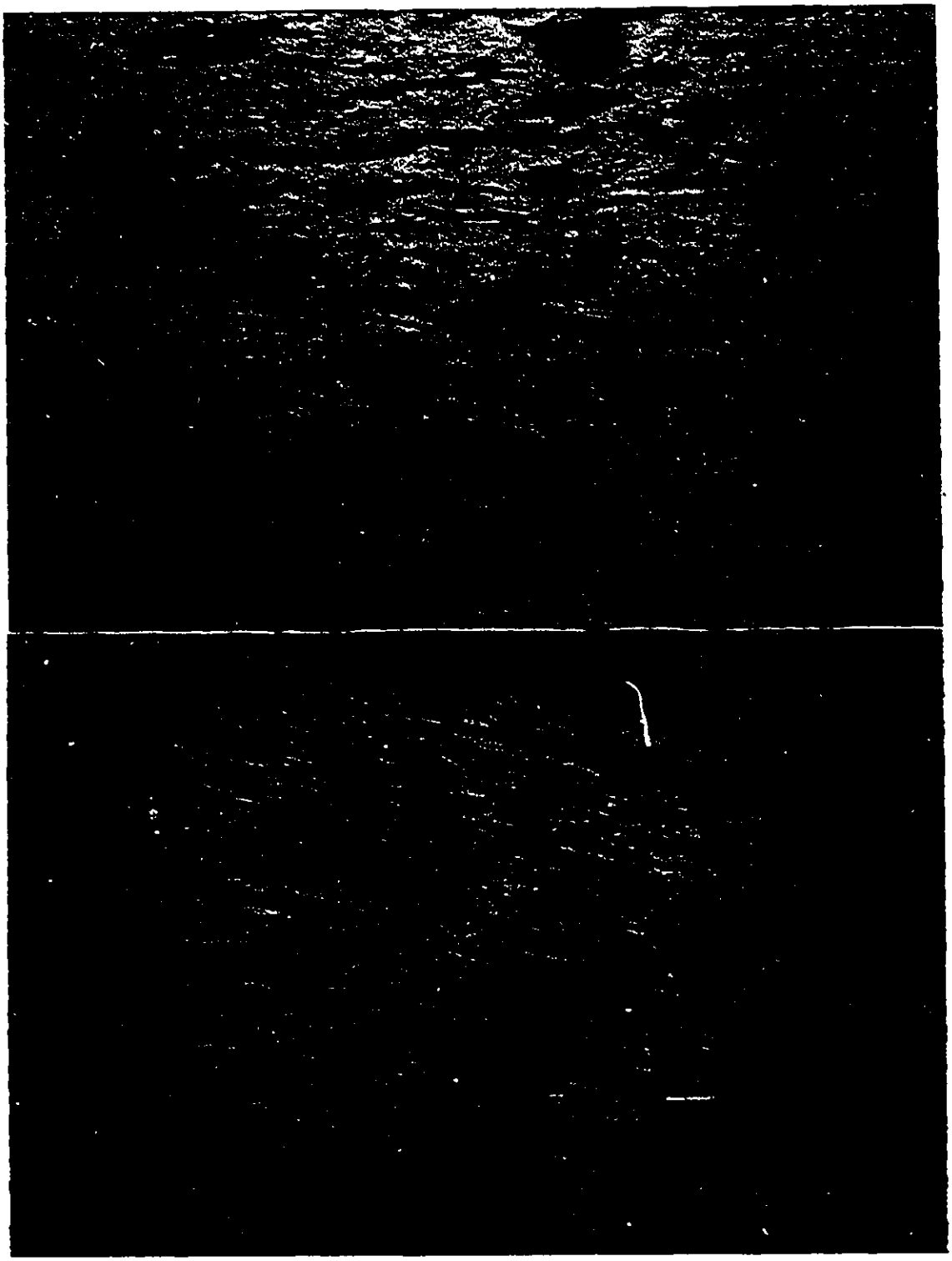


Figure 5. Schematic representing distribution of colored microspheres
○ = represent red spheres infused into the LAD.
● = represent blue spheres infused simultaneously into LCx.
⊘ = represent green spheres infused into the LCx following LAD occlusion.
|← represent extensions of coronary perfusion boundaries.

A



B

Plate 3 Photomicrographs showing aggregates of colored microspheres observed in the LCx (A) and the BWZ (B) following occlusion of the LAD artery. Green microspheres infused into LCx after LAD occlusion. Mag. 250x

RESULTS

Hemodynamic data:

For the nine experiments, mean arterial pressure remained stable (114 ± 5 mmHg; mean ± SD) during the first three minutes of infusion. Mean arterial blood pressure was 112 ± 7 mmHg at five minutes, 104 ± 7 mmHg at eight minutes, and 100 ± 11 mmHg by the ten minute mark, when infusions were concluded. Also monitored in these experiments were blood flow in the LAD and LCx during the infusion period, as well as left ventricular pressure and its first derivative, dP/dt. In these experiments, coronary blood flow and other hemodynamic variables were stable throughout and after the 10 minute infusion period.

Following occlusion of the LAD, mean arterial pressure remained stable in all hearts throughout the period of infusion of the final set of microspheres at 98 ± 13 mmHg. Mean left circumflex flow increased from a baseline 56 ± 7 ml/min to 71 ± 9 ml/min by the third minute of infusion with the final infusate. By the fifth minute of infusion of the final microsphere aliquot, mean circumflex flow was 66 ± 7 ml/min, and 62 ± 8 ml/min at the 8 minute mark into infusion of microspheres. By the ten minute mark, LCx flow was 53 ± 11 ml/min. Aside from three experiments (not reported) where ventricular fibrillation occurred during infusion of the final set of microspheres, there was no significant decrement of left ventricular function in the experiments.

Morphometric measurements:

The distributions of ITZ and BWZ widths were not appreciably different from those illustrated in the previous chapter. For the present investigation, the ITZ ranged from 3487 μm to 7400 μm, with the mean ITZ width being 5377 ± 817 μm; mean ± SD. The BWZ ranged from 1801 μm to 5200 μm, with a mean value of 3358 ± 618 μm. A 2-factor ANOVA was employed to assess the effect of (A) the effect of regional differences Sub-Epi vs Sub-Endo to the observed zone dimensions and (B) the individual hearts. The mean width of the ITZ was 5996 ± 521 μm in subepicardial tissue and 4758 ± 544 μm in subendocardial tissue ($p < 0.001$; 2 factor ANOVA). The mean width of the BWZ was 3673 ± 539 μm in subepicardial tissue and 3043 ± 526 μm in subendocardial tissue ($p < 0.001$; 2 factor ANOVA).

Green microspheres infused into the patent LCx were also found within the baseline boundaries. Prior screening and analysis from thirty randomly selected tissue sections from three hearts comprising both subepicardial and subendocardial regions indicated that the numbers of green microspheres as well the numbers of green/red aggregates within the baseline BWZ region, were not significantly different from the numbers of blue microspheres, and blue/red aggregates respectively (1-Factor ANOVA). These observations suggested that the coronary perfusion interface was receiving a significant compensatory blood supply subsequent to LAD occlusion.

The distribution of the final color of infused microspheres was analyzed as a potential contributing component of microvascular collateral flow within the

occluded LAD bed. A split-plot factorial ANOVA was selected as the procedure of choice to determine the effect of microsphere density as a function of distance from the baseline perfusion boundaries. Analyses were computed based upon the numbers of green microspheres identified within 5 consecutive intervals of 500 μm, to a distance of 2500 μm from the ITZ border in the LAD perfusion field. In addition, the potential contributions of regional differences (Sub-Epi vs Sub-Endo), the individual tissue sections within hearts (nested factor), and finally the separate hearts were also assessed. If significant main effects were revealed, the Scheffé post-hoc procedure was employed for contrasting the levels of the factors.

ANOVA revealed a significant effect based on the density of the final infused microspheres as a function of distance ($p < 0.001$). Specific pairwise comparisons using the Scheffé procedure indicated a moderate statistical difference for the numbers of spheres up to 500 μm from the perfusion interface vs the distance of 1500 μm to 2000 μm from the interface ($p < 0.05$). Highly significant differences were computed ($p < 0.001$) when contrasting the interval corresponding to 2000-2500 μm from the interface, vs all other intervals (Figure 6). Also computed was a significant main effect for hearts ($p < 0.05$), as well as a significant interaction effect between hearts and distance ($p < 0.05$).

Within each tissue section, the locations at which the densities of green spheres as well as green/red aggregates decreased abruptly were recorded. These locations were compared to the ITZ and BWZ boundaries respectively, and identified the lateral extensions of the individual baseline boundaries. The

extended ITZ (E-ITZ) ranged from 4693 μm to 8246 μm, with the mean E-ITZ width being 6693 ± 910 μm; mean ± SD. The extended BWZ (E-BWZ) ranged from 3245 μm to 6545 μm, with a mean value of 4976 ± 709 μm. The mean width of the E-ITZ was 7115 ± 545 μm in subepicardial tissue and 6071 ± 563 μm in subendocardial tissue. The mean width of the E-BWZ was 5420 ± 569 μm in subepicardial tissue and 4531 ± 538 μm in subendocardial tissue. A Student's t-test was applied to contrast the mean widths of the extended ITZ and BWZ boundaries (following occlusion of the LAD) to the mean baseline values. The mean width of the ITZ increased by 24% ($p < 0.01$) and the mean width of the BWZ increased by 48% ($p < 0.01$) These results are illustrated in Figures 7 and 8.

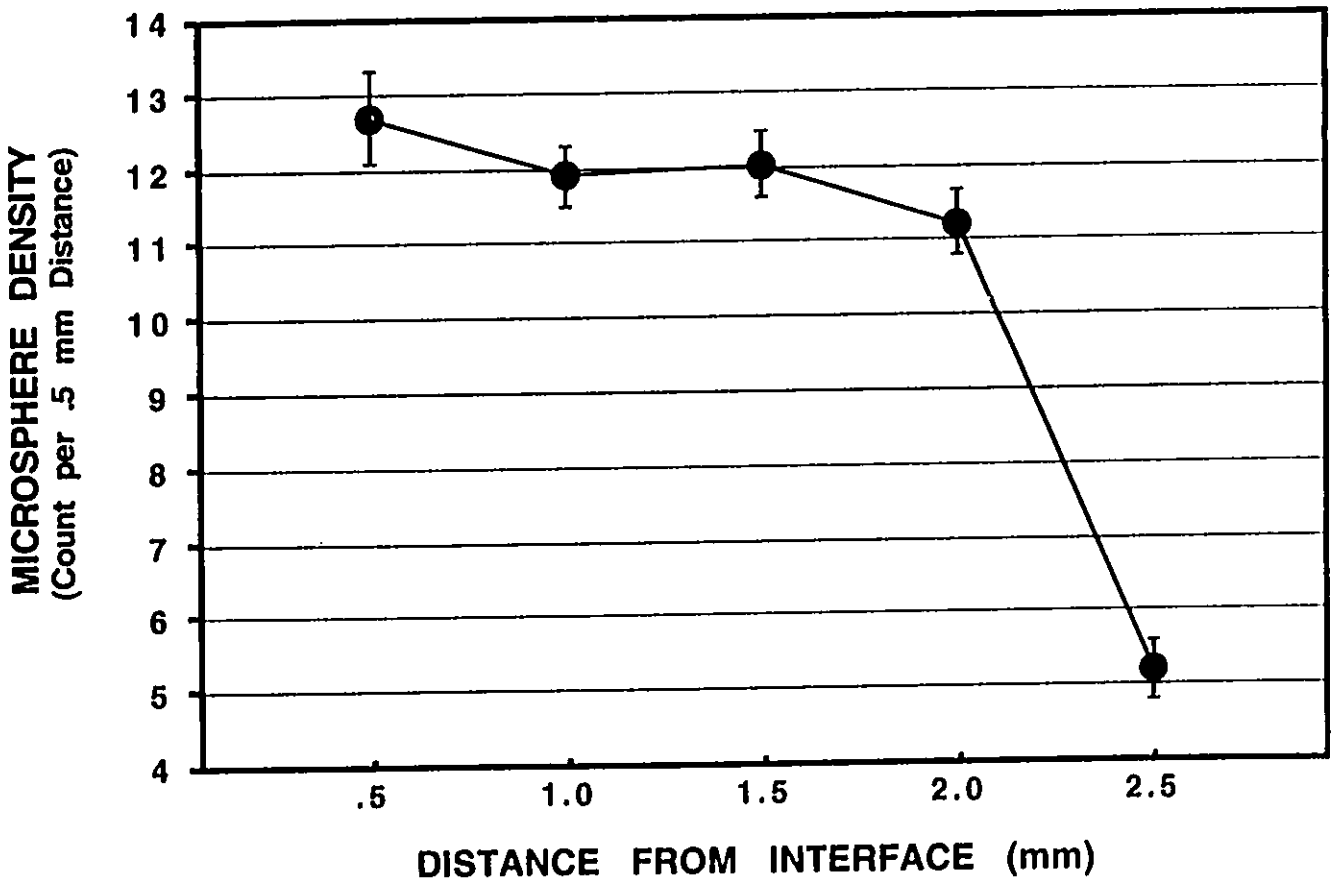


Figure 6. Density of final injected microspheres as function of distance from the perfusion interface. ANOVA revealed an effect of distance ($p < 0.001$), to the density of the latter infused spheres. Specific pairwise comparisons indicated a moderate statistical difference between the interval width from 0.0mm to .5 mm vs that from 1.5mm to 2.0mm ($p < 0.05$). Highly significant differences were computed ($p < 0.001$) when contrasting the interval width from 2.0mm to 2.5mm vs all other intervals. Points represent mean ± SEM.

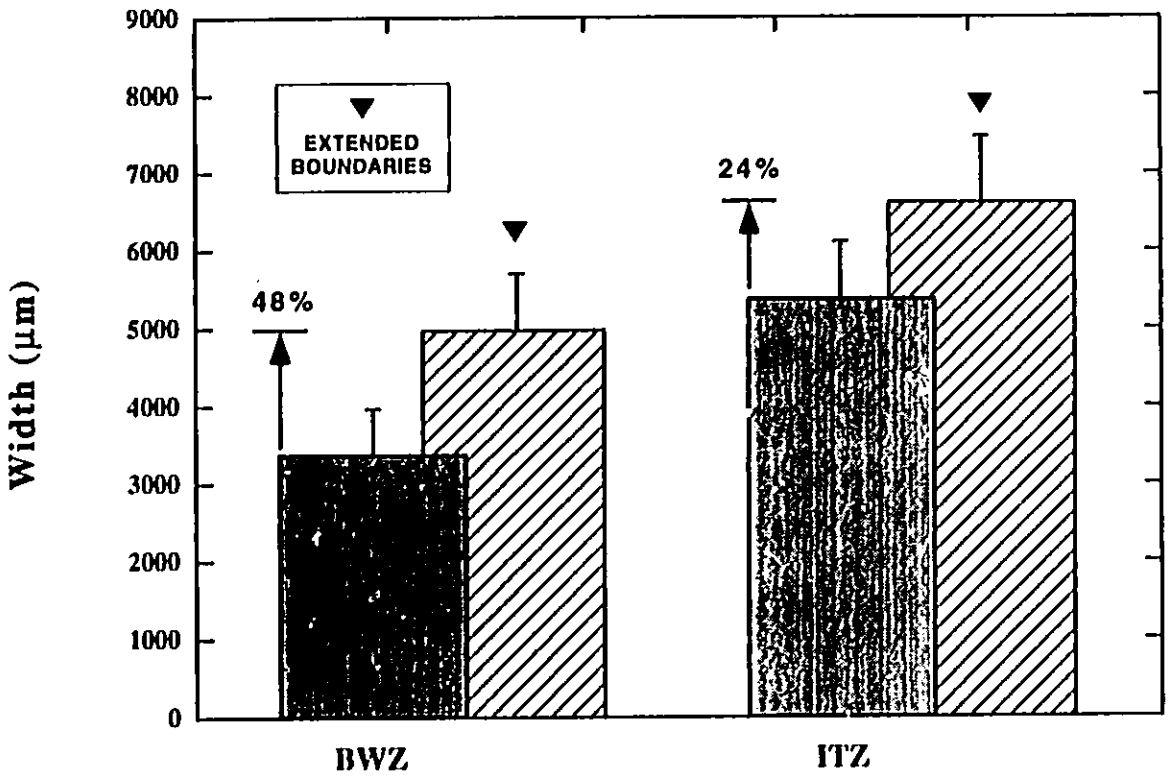


Figure 7. Graphical representation of BWZ and ITZ boundary widths (mean ± SD) and their lateral extensions. Analysis indicated significant increases ($p<0.01$) of 48% and 24% respectively into the LAD of these boundaries.

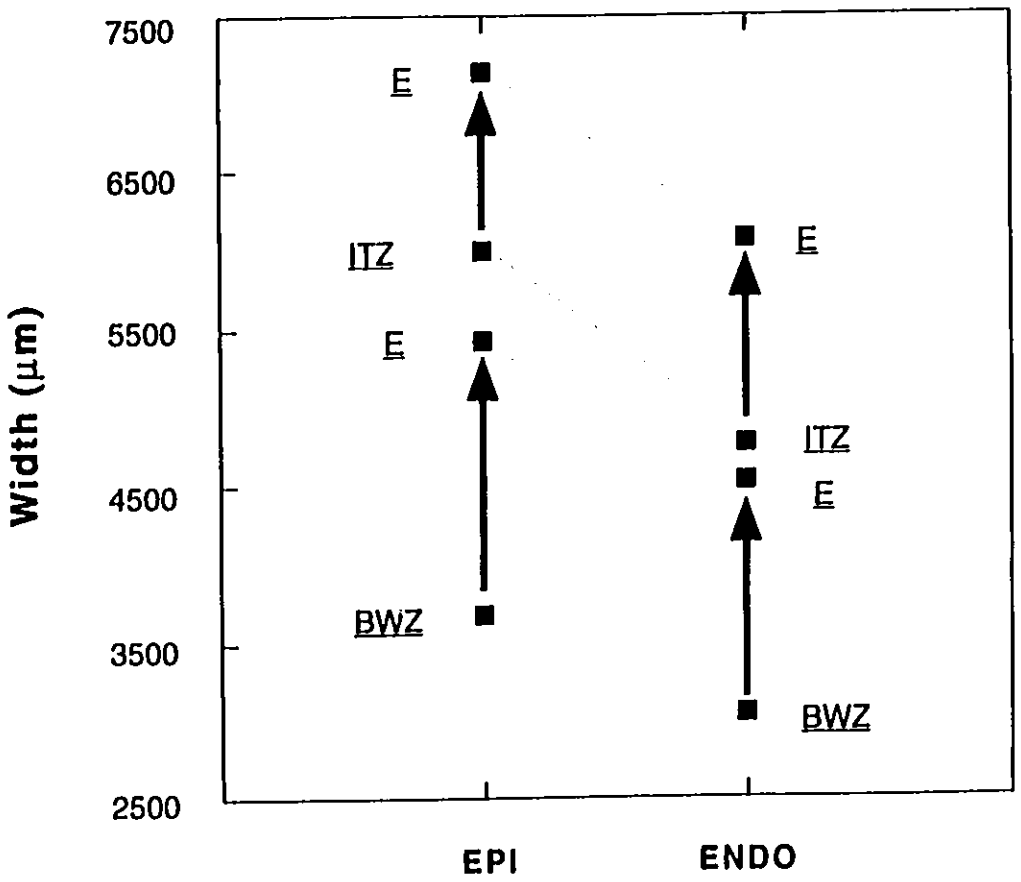


Figure 8. Graphical representation of the effect of regional differences (EPI-ENDO) on BWZ and ITZ boundaries and their lateral extensions (depicted by "E") into LAD region. All boundaries were significantly greater in subepicardial regions than in subendocardial regions ($p<0.001$)

DISCUSSION

The existence and potential functional importance of collateral vessels in the mammalian coronary circulation is well recognized (see Cohen, 1985; Schaper et al., 1988). However, a widely accepted belief has been that the larger, epicardial vessels, and to a lesser extent, a network of intracoronary arteriolar connections (Schaper, 1971, 1979) constitute the dominant sources of redundant blood flow, particularly in pathologic hearts with coronary obstructive disease.

Our preliminary investigation described in the previous chapter indicated that myocardial capillaries at the junction of coronary perfusion territories were supplied with blood from both coronary arteries. These findings resulted from application of our colored microsphere technique to microscopically label adjacent perfusion territories of coronary arteries in the *in situ* working canine heart. In addition, the width of this zone of dual perfusion was found to be significantly greater in subepicardial tissue than in subendocardial tissue. These results are, furthermore, consistent with previous anatomic investigations having indicated microvascular anastomotic pathways in the human and canine coronary circulations (eg. Brown, 1965; Fulton and Thomas, 1965; Baroldi and Scomazzoni, 1967; Bassinthwaighte et al. 1974; Grayson et al., 1974). The preponderance of microvascular anastomoses has been identified as intracoronary, and thus connecting branches of the same artery. In addition, intercoronary microvascular anastomoses connecting perfusion territories supplied by different parent coronary arteries have also been described.

Those studies based on post-mortem silicone rubber injection technique on the other hand proposed that capillaries derived from any coronary artery were discrete and thus lacked the capability to produce a gradient of perfusion at the border of an ischemic region. However, the inherent limitations of the "Microfil" method as applied to the coronary vasculature were extensively discussed in the introduction to Part 1 of this dissertation. Thus, results produced by this technique are likely not definitive.

Arterial collateral vessels are traditionally depicted as having an "H" configuration such that there is no appreciable flow in the collateral (the cross arm of the "H") without a pressure difference between the two arteries (Cohen, 1985; Schaper, 1971). These collaterals can provide an important source of compensatory blood flow to large perfusion territories when a substantial coronary branch is obstructed. However the findings of Przyklenk et al. (1986), and our preliminary work described in the previous chapter have led us to propose a somewhat different description of blood supply to tissue along the interface of coronary perfusion fields. We suggest that the dual arterial supply to interface tissue may often take the form of a "Y" configuration, with the arms of the "Y" being the extensive microvascular collaterals that have been observed in past anatomical studies (eg. Brown, 1965; Fulton, 1965; Baroldi and Scomazzoni, 1967; Grayson et al., 1974). Since each arm of the "Y" channel receives blood from different parent arteries, capillaries at the base of the "Y" have dual sources of perfusion. This anastomotic pattern would account for the presence of both colors of microspheres in capillaries along the interface under normal perfusion conditions.

Obstruction of an artery supplying one arm of the "Y" would not stop flow to tissue fed by capillaries at the base of the "Y". During an upstream coronary obstruction, one of the arms of the "Y" could function as a collateral pathway due to the large pressure gradient across the arms of the "Y". In other words, with a higher order obstruction, the normal "Y" pathway would function as a traditional collateral "H" pathway, but at the microvascular level. Such a model is consistent with our present results.

Presently, our colored microsphere technique has been utilized only to demonstrate the presence of watershed zones along the boundaries of major coronary perfusion territories. However, on the basis of available anatomical data it is reasonable to hypothesize that such a pattern of arterial perfusion might exist elsewhere in the coronary circulation, and not merely along the junction of perfusion fields. Therefore, we speculate that coronary capillaries throughout the heart possess redundant blood supply provided by Y-like microvascular anastomoses.

The principal discovery of the present investigation is that acute coronary occlusion can alter the zone of dual arterial supply by creating a shift or extension of the perfusion interface into the occluded, acutely ischemic region. These results are evidenced by the distributions of the latter color of injected microspheres relative to the positions of the previously injected microspheres infused into the two coronary branches under normal perfusion pressure conditions.

The zone of microvessels containing microspheres of both initial colors was limited exclusively to the interface region. Within the interface, capillaries were

observed containing one or the other of these colors. This region has been described previously as the ITZ. Within the ITZ was a region (the BWZ) where certain capillaries contained both initial microsphere colors. The admixture of different colored microspheres within the interface undoubtedly resulted from microvascular anastomoses, and not from higher order collateral pathways. Higher level anastomoses would have distributed alternate color spheres throughout wide areas of the perfusion territories of both coronary branches, and not simply within the interface. Following occlusion of the LAD, microspheres of the latter infusion, which were distributed throughout the LCx, were also found in significant numbers within these baseline boundaries. The perfusion territory of the patent LCx branch could now be defined based on the uniform density of the final infused microspheres which extended the ITZ, as well as the BWZ into the LAD perfusion territory. Capillaries in the LAD region adjacent to the interface were often found to contain numerous green microspheres as well as red/green sphere aggregates.

Due to the presumably large pressure gradient generated from occlusion of the LAD, collateral flow would be expected to occur at all levels of the circulation where collateral vessels are found to exist. Higher order, larger collaterals which are the only types of vessels that certain investigators believe to exist (eg. Factor et al. 1982; Harrison et al. 1986; Murdock et al. 1983) on the other hand, would be expected to cause microspheres infused into the patent artery to distribute essentially uniformly within the perfusion territory of the occluded vessel. In fact, such a pattern would follow the "typical" distribution pattern of radioactive

microspheres that is observed in retrograde flow studies in ischemic myocardium when tissue with any access to perfusion is eliminated as sample contamination by use of the "shadow technique" (eg. Kirk 1980; Murdock et al. 1983).

Microvascular anastomoses crossing the interface would, however, introduce an additional component of collateral flow in this region. This would be apparent by the greater density of the latter injected microspheres from the patent vessel near the interface than in tissue more removed from the interface. Indeed, our present results strongly suggest a microvascular component of collateral flow, since the density of the third set of microspheres localized in the ischemic region was significantly greater near the interarterial interface than in tissue further removed from it. The finding that at a distance of 2500 μm and beyond the ITZ, the density of microspheres infused following LAD occlusion decreased precipitously compared to tissue closer to the interface, further indicates a lesser contribution of larger upstream collateral vessels to the observed microsphere distribution patterns.

Thus the results of studies which have employed the shadow technique (eg. Hirzel et al., 1976; Kirk, 1980; Murdock et al. 1983) claiming that residual antegrade perfusion during retrograde flow diversion was due to contamination from overlapping, normally perfused tissue may be innovatively reinterpreted to support our view that microvascular anastomoses are indeed responsible for the residual antegrade flow.

According to the distribution patterns of colored microspheres in acutely ischemic canine myocardium, microvascular anastomoses appear to be largely

responsible for the extension of the perfusion field of the patent coronary vessel into the ischemic territory of the obstructed artery. It appears unlikely, however that the functional anatomic limit of these anastomoses extends far beyond the periphery of the ischemic region. It is thus at the periphery of the ischemic region where the greatest benefit of microvascular anastomoses should be derived. With more distal sites of coronary artery occlusion, the periphery comprises a greater proportion of the mass of the acutely ischemic region. Studies which have investigated this phenomenon indicate that the infarcted region, expressed as a percentage of the ischemic region at risk, decreases as the total area of vulnerability becomes smaller (White and Bloor, 1981; Becker et al., 1983; Gumm et al., 1988). Thus, our observations do support a potentially significant role for microvascular collaterals in protecting relatively small regions of myocardium from ischemic damage.

In summary, the present study indicates for the first time that the perfusion interface is both labile and potentially responsive to manipulation during conditions of acute coronary occlusion. Furthermore, the survival of this borderzone of intermediate perfusion may be of importance following small artery obstruction. A dual arterial supply to microvessels along the border of coronary perfusion territories could provide a compensatory blood supply, thus serving a role in protecting myocardium along the junction of perfusion fields from microemboli entering or forming in the coronary circulation. Recent studies suggest that coronary microembolization primarily due to platelet aggregation is a clinically important entity (El-Maraghi and Genton, 1980; Davies et al., 1986;

Fuster et al., 1987; Tofler et al., 1987). Coronary embolism is usually clinically silent (Roberts, 1978), which may in fact be the result of the dual microvascular supply along the coronary perfusion interface. Larger numbers of emboli occluding microvascular elements more removed from the perfusion interface increases the likelihood of creating pockets of focal ischemia, which might precipitate cardiac arrhythmias. Although a multitude of phenomena probably contribute to the syndrome of sudden cardiac death, which is presumably caused in large part by premature ventricular depolarizations (Fuster et al., 1987), induction of platelet aggregation in the coronary circulation could increase the probability of sudden cardiac death, particularly in susceptible individuals with unstable angina (Davies et al., 1986). Additional support for the role of platelet emboli in provoking such events derives from studies of pigs where it was determined that coronary platelet aggregation significantly increased the occurrence of arrhythmias leading to sudden death (Jorgensen et al., 1967).

Further investigations are clearly warranted to determine how myocardial watershed zones could be altered pharmacologically, thereby providing a rational basis for new therapeutic modalities aimed at salvaging ischemic myocardium.

PART B: CAPILLARY FLOW VECTORS IN MYOCARDIUM

INTRODUCTION

HISTORICAL OVERVIEW:

The earliest efforts at distinguishing vascular continuity between arteries and veins were provided by Marcello Malpighi in 1661. Using double convex microscopic lenses, Malpighi was able to see blood passing from arteries to veins in a variety of organ beds, including the frog lung. He described networks of minute blood vessels in communication from artery to vein possessing little demonstrable order. Malpighi further proposed that the blood always flowed through these tubed vessels, and that it did not pour into spaces or pass via seepage through the parenchyma of organs as had been believed by many through the time of William Harvey. Malpighi's lenses were still not powerful enough to do more than characterize blood as a liquid consisting of an almost infinite number of particles. It remained for Anthony van Leeuwenhoek, in 1674, to actually see the cells flowing in the capillary streams of tadpoles using his own system of precision grounded lenses.

Descriptions of microcirculatory networks on a fundamental structural basis were provided by Marshall Hall, in 1831. He described arterial vessels as those that progressively subdivided into smaller branches. Veins, on the other hand continued to enlarge from successive convergences, and the terminal vessels, the capillaries, were found to be discrete from these larger vessels. Although he discovered that there were progressive arborizations and confluences among them,

capillary diameters appeared to remain constant. Hall intuitively ascribed functional characteristics to these terminal vessels, where their numbers and distributions in individual tissue beds could be altered in response to changing demands.

The complexity of the coronary microcirculation began to be appreciated from the pioneering work of Meigs in 1899. He described the enormous number of minute vessels comprising the microcirculation based upon his dye injection experiments of human hearts at autopsy. Shortly thereafter, the capillary network in the mammalian heart was described in somewhat greater detail by Spalteholtz (1907) and Nussbaum (1912). Their significant observations spearheaded future investigations concerning microvascular topology and flow pattern, adapted to the functioning of the heart under normal as well as pathologic conditions,

However, it was only after Wearn (1928) employed injections of India ink suspensions in gelatine into the coronary vessels of isolated perfused, and beating hearts that a satisfactory quantitative method became readily available for demonstrating the microcirculatory patterns of the myocardium. His important contributions set the stage for the direct qualitative and quantitative investigations of the coronary microcirculation that were to follow.

Theoretical Studies:

The epoch-making studies of August Krogh (1919) first revealed the significance of the terminal vascular bed to the nutritional and oxygen requirements of muscle tissue. Krogh proposed that oxygen transport was dependent upon the

numbers of capillaries localized within the tissue. Based upon his observation that capillaries were uniformly distributed in tissue sections cut perpendicularly to the muscle fiber axis, Krogh suggested that each capillary be considered as running in parallel to the muscle fiber, and supplying a concentric cylinder of tissue with the capillary in the center of the cylinder. Furthermore, this "Krogh" cylinder was completely independent of other capillary-tissue cylinders. The average cylinder radius could simply be determined by counting the number of capillaries per cross-sectional area, and by dividing the cross-sectional area by the number of capillaries. The cylinder radius represented the maximum diffusion distance of oxygen from the capillary. With the assistance of the Danish mathematician Erlang, they obtained an analytical solution that provided quantitative values for oxygen tension in tissue, through the now famous Krogh-Erlang equation. Krogh's model, due to its relative simplicity, has been widely used in physiological studies to assess oxygen supply conditions.

Notwithstanding, the equation is based on a number of assumptions and limitations. In his detailed review of the Krogh cylinder model, Kreuzer (1982) enumerates that among these simplifications are (1) the idea that only radial diffusion into the tissue exists, and (2) the equation assumes that oxygen consumption did not depend upon the local PO_2. Krogh further assumed (3) that the capillary wall presented no resistance to oxygen diffusion (4) that there was no facilitation to diffusion by myoglobin, and (5) that capillary flow was constant with no interchange of oxygen between adjacent cylinders.

Although Krogh's model can describe the conditions for gas exchange accurately only in the case of a mass of tissue within which adjacent capillaries lie in parallel, begin and end in the same plane and carry blood flowing in the same direction (i.e. concurrent capillary flow), the model has proved to be an extremely useful conceptual tool for the modeling of oxygen transport to tissue.

Following the original work of Krogh, other investigators have also considered diffusion processes of oxygen and metabolites through tissue. A.V. Hill (1928) extended Krogh's steady state model to the unsteady state. The model of Hill was developed to describe the diffusion of oxygen into isolated nerve preparations from the solution in which those nerves were bathed. The Hill model characterized oxygen supply uniformly from the periphery into the muscle fiber, whereas the Krogh model described oxygen supply outward into the tissue from a single capillary.

Rakusan (1971) described changes in myocardial PO_2 in relation to various oxygen determinants based upon the Krogh-Erlang equation. Using graphical analysis, he considered the individual effects of factors such as oxygen consumption, myocardial blood flow, capillary radius, diffusion distance, and oxygen content within the capillaries on PO_2 at the periphery of the tissue cylinder. The most important factor in the PO_2 was the myocardial oxygen consumption. Rakusan found that the cylinder radius was an important factor only in cases where it was increased, as in cardiac hypertrophy.

A traditional approach over the years for assessing myocardial capillary supply from tissue cross sections has involved measurements of capillary density, i.e. the number of capillaries per unit of cross sectional area. However, this measurement has been shown not to provide information regarding variability in capillary supply within the area from which capillary density is measured. In fact, tissue oxygen supply has been shown to be compromised as the heterogeneity of capillary spacing increases (Turek and Rakusan, 1981; Rakusan et al., 1985). Employing the method of concentric circles, Turek and Rakusan (1981) derived the average Krogh radius from this method, and evaluated the heterogeneity of capillary spacing and its effect on tissue oxygenation in normal and hypertrophic rat heart.

Since the number of capillaries is often related to the number of muscle fibers present, another commonly reported index of capillary supply is the fiber-capillary ratio. This index, however, ignores the size of the individual muscle fibers. It is possible, for example, to decrease the fiber-capillary ratio by a factor of four and yet have the distance between any two capillaries on cross section remain unchanged.

The term intercapillary distance (ICD) has also been used as an index to quantify the diffusion distances from a random point in the tissue space to one or more capillaries. The value is normally derived from the straight line measurement from the center of one capillary to another from either cross or longitudinal sections. Thus, the shorter the intercapillary distance the more favorable would be the conditions for oxygen diffusion to tissue. One half of the ICD may be taken as

the radius of the Krogh cylinder, although this value may not be symmetric when the distance from a reference capillary is not uniform. A number of theoretical geometric models involving capillary stacking patterns have been used to relate the capillary density to the intercapillary distance. These include hexagonal, rectangular, or triangular stacking of capillaries, with the capillaries located at the apices of these arrays. In cardiac muscle, intercapillary distances have been found to be more compatible with a log normal than the normal "Gaussian" distribution (Renkin et al., 1981; Rakusan et al. 1986). The index of central tendency in a log normal distribution is the median with its variability optimally described by the standard deviation of the log transformed ICD's.

A recent theoretical model of tissue capillary supply has also been proposed. By associating each capillary profile within a Voronoi, also known as Dirichlet tessellation, a polygonal boundary can be formed around a region of tissue closer to the enclosed capillary than any other (see Figure 9). Such boundaries are also found to appear in diverse physical phenomena: eg. geography (Rhynsburger, 1973), ecology (Ripley, 1981), crystallography (Gilbert, 1962), and others. This model was first applied to coronary capillaries on cross-section by Hoofd et al. (1985). It partitions the tissue plane into an irregular lattice pattern where the areas of individual polygonal tissue regions surrounding capillary profiles are referred to as "capillary domains". These domains are considered to represent the respective tissue supply regions. From the domain area, the equivalent radius of the Krogh cylinder with the same area may be calculated. Since the distribution of these radii is also found to be log normal, the variability is optimally characterized

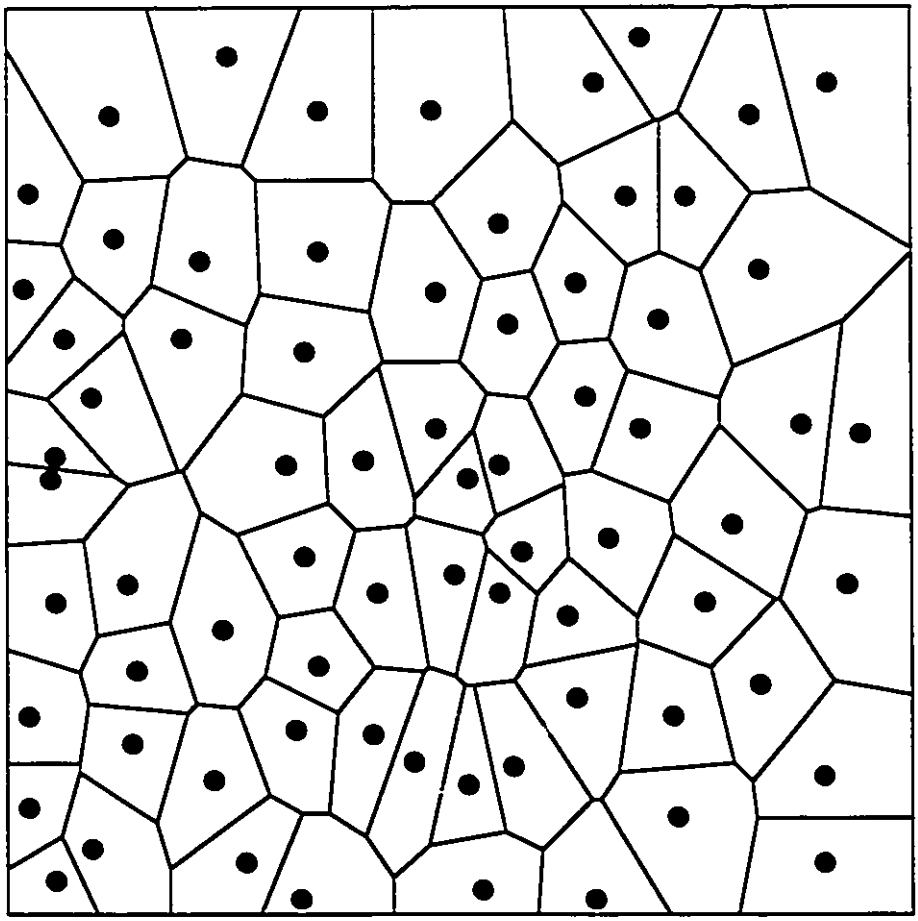


Figure 9. Typical computer printout of capillaries represented by shaded circles and their individual capillary domains. The areas subtended by these polygonal regions of tissue are closer to the enclosed capillaries than any other.

by the logarithmic standard deviation (SDlog). This measure of variability further serves as an index of the heterogeneity of capillary spacing.

The assumption of straight and parallel capillaries with uniformly concurrent and steady flow as depicted by the Krogh model is, no doubt, an extreme idealization. Not surprisingly, other theoretical models of tissue oxygen supply have also been considered. The first steps taking into account capillary interaction were made by Diemer (1965), Bailey (1967), and Reneau and Knisely (1971) who considered different models of mass transfer under countercurrent flow conditions. Diemer (1965) first proposed that a thorough analysis of transport mechanisms must take into account not only blood flow through individual microvessels, but also the influence of capillary arrangements on tissue oxygenation. In this connection, the direction of blood flow in neighboring capillaries was considered important i.e. the presence of concurrent or countercurrent flow. It was further suggested that countercurrent flow between adjacent capillaries provided a more homogenous supply of oxygen to the individual cells than concurrent flow conditions might. Metzger (1969) also considered the number of situations in which adjacent capillaries exhibited either concurrent or countercurrent flow direction a physiologically important parameter. In his model, oxygen diffusion from 2 and 3-dimensional capillary structures in the form of square or cubic lattices was considered. An important feature of this model was the possibility to study the impact of heterogeneous capillary perfusion and flow direction on the distribution of oxygen in the tissue. Metzger found that for a 2-dimensional tissue model, the concurrent system was least favorable, and the countercurrent system

most favorable for oxygen extraction. Metzger (1976) later applied the 2-dimensional square lattice model to study the effect of spatially inhomogeneous oxygen consumption on oxygen distribution in the tissue.

Grunewald (1968) developed a model consisting of 4 parallel capillaries situated along the side of a rectangular "parallelepiped" or microcirculatory unit (MCU) the inlets and outlets of different capillaries situated in different planes that allowed for consideration of concurrent, countercurrent and, asymmetric (arterial inflow and venous outflow directed opposite with respect to the middle portion of an adjacent capillary) flow conditions. Grunewald and Sowa (1977) utilized the MCU model applied to skeletal muscle at rest, and concluded that the concurrent MCU and asymmetric MCU models accounted for 61% and 39% of cases respectively, while the countercurrent MCU configuration was virtually absent. This model was also applied to simulate oxygen transport in the heart. In their analysis of oxygen supply in the normoxic rat heart, Grunewald and Sowa (1978) suggested that MCU's depicting a complete concurrent flow arrangement would best explain PO_2 distribution values in myocardium.

Lübbers (1976), on the basis of studies from models obtained by means of accumulation of oxygen histograms in various tissues including the heart, described two favorable patterns of capillary distribution (see Figure 10): (2) symmetrical antiparallel input and output (the countercurrent flow model) and (3) an asymmetric arrangement (similar to the model of Grunewald and Sowa, 1978, described above). The countercurrent arrangement was seen as having a definite

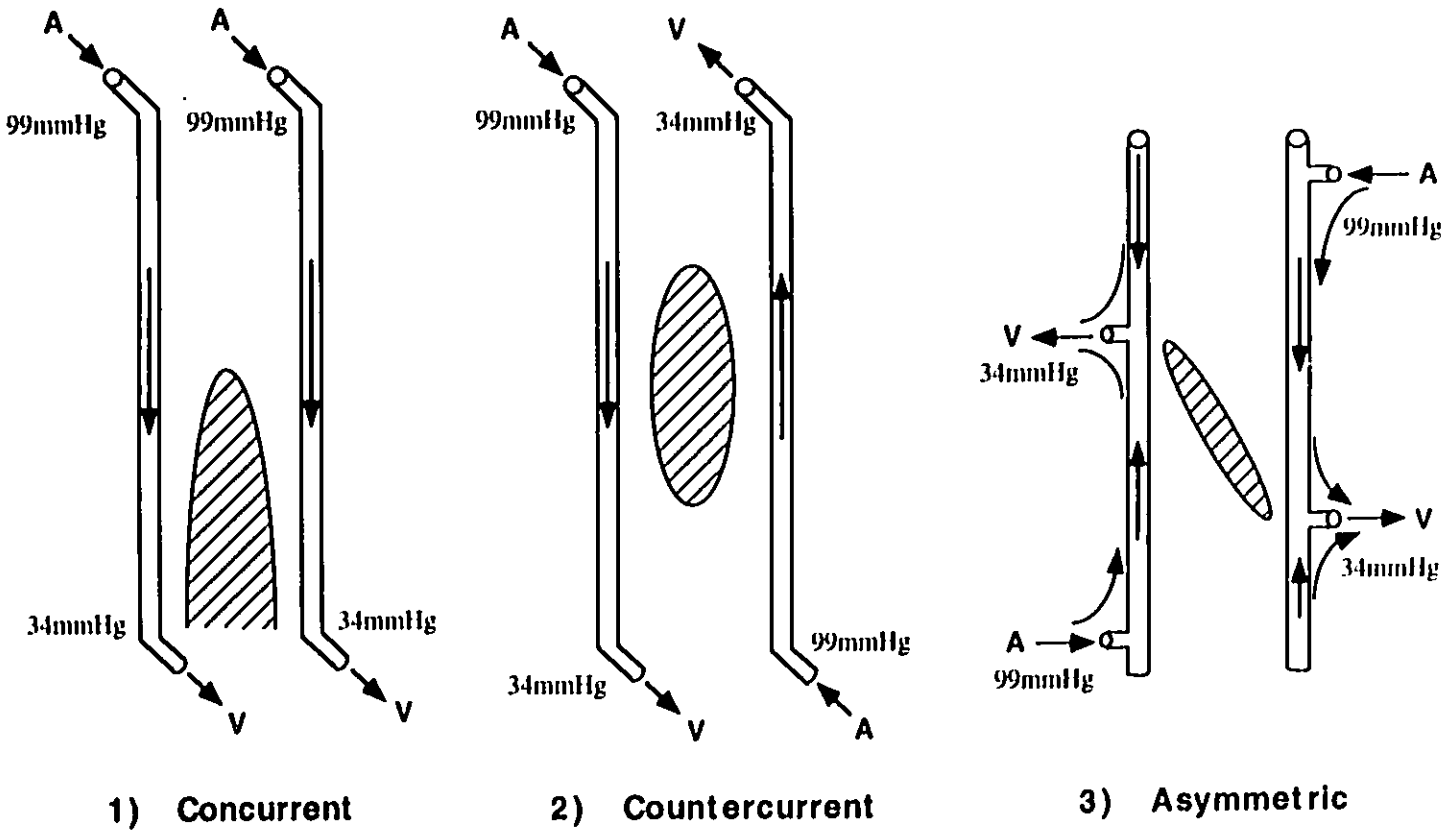


Figure 10. Schematic depiction of three patterns of capillary flow distribution modified fromthe model of Lubbers (1976). Arrows indicate direction of capillary flow. In concurrent flow (1), capillaries are symmetric and have parallel arterial (A) input and venous (V) output. In the countercurrent flow scheme (2), capillaries are symmetric, but have opposite input and output. In the asymmetric pattern (3), input and output are not symmetrically distributed. Potentially jeopardized regions of tissue, i.e., lowest tissue oxygen pressure are illustrated by the shaded areas.

advantage over the concurrent capillary pattern, since arterial PO_2 would be more efficiently utilized. However, with this flow arrangement it was possible for oxygen to be shunted by diffusion directly from the arterial inflow to the opposite venous outflow, potentially depriving more distal tissue of oxygen. The second, and in Lübbers' opinion, more efficient system appeared to be an asymmetric structural pattern, where the oxygen diffusing out of one capillary was still available to a second, adjacent capillary, some distance removed from a potentially jeopardized area of capillary venous outflow.

Popel (1980) applied an analytical solution to a simple case of heterogeneous capillary flow represented by alternating layers of capillaries with concurrent flow. Asymmetry in oxygen distribution was introduced by considering different velocities of blood in the alternate capillary layers, different inlet capillary oxygen tensions, and different capillary hematocrits. Symmetric and asymmetric distributions of oxygen concentration between the layers were compared. Solutions for the symmetric case were very close to the corresponding solutions of the Krogh cylinder model, but an increase in the degree of asymmetry led to diminution of the mean oxygen tension.

Wieringa et al. (1982) developed a stochastic three-dimensional network model of straight parallel capillaries in the heart in a hexagonal array with randomly placed interconnections between adjacent capillaries as well as randomly generated locations of arteriolar inflows and venular outflows. In this model, the range of the ratio between the number of concurrent and countercurrent flow

flow patterns was 0.90 to 1.95 with a mean ratio of 1.32, indicating that on average 57% of the situations were concurrent and 43% countercurrent. In total, eight different capillary network situations were simulated, by varying the number and flow distribution of arteriolar sources. The flow through individual capillaries was calculated for each situation. These authors' calculations indicated the presence of a large heterogeneity in the capillary flow rate between adjacent capillaries, suggesting that the anatomy of the capillary network may give rise to areas of profound underperfusion even under steady state conditions.

From this brief survey of theoretical geometric models of microvascular flow pattern, it is apparent that inconsistencies and conflicting results exist. On the other hand, it is not entirely surprising given the numerous assumptions that need to be made. For any theoretical model, the availability of basic and reliable entry data has proven to be crucial toward a detailed understanding of oxygen transport. For the myocardium, such data have been particularly difficult to obtain.

Anatomical Studies:

Over the years a number of *in vitro* techniques have also been applied to describe the morphological pattern of the terminal vascular bed in myocardium. Wearn (1928) was the first to employ a reliable method (India ink in gelatine) for visualizing the coronary microcirculation. Using this technique, Shipley et al. (1937) considered capillary supply in normal and hypertrophied rabbit heart at

various ages. Roberts and Wearn (1941) provided the first quantitative descriptions of capillary supply in normal and pathologic human hearts.

Rakusan and Poupa (1963) employed similar methods to provide quantitative descriptions for changes in capillary density, capillary/fiber ratios, and diffusion distances in rat heart during postnatal development, and later, in hearts of aged rats.

Brown (1965) following in the footsteps of Roberts and Wearn (1941) utilized India ink injections in the coronary arteries of isolated and perfused beating hearts to describe microvascular networks in 6 different species. Brown described the terminal arteriole as penetrating a muscle bundle at various oblique angles, and furnishing capillaries that often appeared to run in opposite directions and drained into venules quite a distance apart. He also noted that not all the capillaries from a given terminal arteriole emptied into the same collecting venule. Using similar techniques in the rabbit heart, Ludwig (1971) also noted that terminal arterioles approached the muscle fibers at an oblique angle, and furnished capillaries at various levels as well as in different directions in the tissue.

Bassingthwaighte et al. (1974) injected silicone elastomer (Microfil) into the left anterior descending branch of the left coronary artery in the beating dog heart. Following clearing of the tissue with increasingly concentrated ethanol and in methyl salicylate, the vasculature could be visualized by reflected or transmitted light microscopy. These authors indicated that the coronary capillary system exhibits no clearly definable beginning or end but instead appeared to be sequentially supplied by terminal arterioles and drained by collecting venules. The

overall anatomic arrangement of the capillary beds appeared to provide a basis for concurrent flow direction in neighboring capillaries. Nevertheless, the authors commented on the difficulty in predicting the probable pathways and flow directions that red blood cells may follow.

Other forms of corrosion casting have also been utilized to study the architecture of the microcirculation in myocardium (eg. Grayson et al. 1974; Phillips et al. 1979; Tomanek et al. 1982; Potter and Groom, 1983). These techniques involve injection of a suitable compound into the vascular bed. After hardening, the compound becomes rigid, preserving the structure of the microcirculation. The tissue component is digested away, leaving only the microvascular cast which may be examined microscopically. With these techniques, it is readily observed that capillaries exhibit a primarily parallel alignment which tend to follow previously observed myocyte contours quite closely. Nevertheless, numerous capillary loops and a high degree of capillary branching could also be observed.

A variety of histochemical techniques have also been used by investigators to identify the coronary microvasculature. The important contributions of Wearn (1928) in identifying capillaries based on injections of India ink suspensions were noted earlier. Another common method used involves the histological impregnation of capillaries. A detailed analysis of various histochemical methods was published by Hort and Hort (1958). Typical reactions that have been employed in experimental work include Periodic acid-Schiff (PAS), alkaline phosphatase (AP) activity, and occasionally silver methenamine staining. In addition, capillaries may be examined from histological sections based upon counts of endothelial cell

nuclei (Linzbach, 1947). This latter method, however, is subject to considerable error as endothelial cell nuclei may be difficult to distinguish from the nuclei of fibroblasts. Further, the number of nuclei does not provide a direct count of the number of capillaries. Another method is to directly visualize red blood cells within the microcirculation. This method is also subject to considerable error, as red cell density is affected by flow velocity, network geometry and capillary hematocrit.

A novel histological staining method introduced by Lojda in 1979, stains capillaries differentially for the activities of alkaline phosphatase (AP), positive in the arterial portion of the capillary, and dipeptidylpeptidase (DPP IV) which is present in the venous portion. This double-staining approach has been successfully applied for obtaining reliable quantitative data specific to coronary capillary geometry (see Batra et al. 1989; Batra et al. 1991a,b; Batra and Rakusan, 1992).

Recently, a variety of immuno-histochemical techniques that label structural glycoproteins found in epithelial basement membranes, such as fibronectin and laminin have been used to identify capillaries (e.g. Borsi et al. 1987; Gobel et al. 1990). Histochemical staining of the capillary vasculature has also recently been employed using the isolectin, Griffonia simplicifolia (eg. Hansen-Smith et al., 1989; Spinale et al., 1992). These investigators reported that the lectin was more sensitive than other histochemical methods for determining capillary density and structure. In addition, this lectin has been successfully used as a fluorescent tracer for microcirculatory vessels (Hansen-Smith et al., 1988).

Intravital Techniques:

While histological methods have been helpful in describing the topology of the coronary microcirculation they are not particularly useful in characterizing the direction of blood flow therein. In contrast, intravital microscopic techniques have allowed for the actual visualization of the coronary microcirculation. Fabel (1968) was the first to employ this technique in his investigation of the microcirculation in the isolated perfused, beating rabbit heart. Fabel noted that while neighboring capillaries usually ran in parallel, their flows were often observed running in opposite directions. He also described the staggered pattern of arteriolar inputs at various depths within the muscle tissue.

In 1971 Tillich and coworkers studied the microcirculation of the beating heart by transilluminating it from the atrial cavity. Their technique, however, could not be applied to investigate the ventricular microcirculation because of the thickness of the ventricular wall and because of focusing problems resulting from tissue movement. Tillmans et al. (1974) studied the capillary bed in canine and turtle myocardium using a refined transillumination technique. By transmitting light through a 20-gauge needle that was inserted underneath the superficial layer of the dog ventricle these investigators were able to observe the anatomical arrangement and functional behaviour of capillaries in the dog heart. Among their observations it was noted that the capillary arrangement appeared to be predominantly parallel and concurrent, however, occasional countercurrent flow loops were also evident.

In order to minimize any traumatic influences on experimental conditions, Steinhausen et al. (1978) developed an epi-illumination technique combined with video microscopy to study the microcirculation of the epimyocardial layers of the beating rat heart under normal and hypoxic conditions. These investigators inserted 5 steel needles into the outer layer of the ventricle to restrict movement and injected a plasma label to improve contrast. They reported a mixed countercurrent flow pattern, with blood flow in 56% of capillaries being concurrent, and the remaining 44% exhibiting countercurrent flow direction.

Similar techniques were applied by Tyml et al. (1981) to describe the structure of a long stretched capillary network in sartorius muscle. Flow directions were noted in their figures. Their network was used to draw seventeen lines at uniform distances (333 μm) perpendicular to the main orientation of the capillaries. The number of concurrent to countercurrent flowing capillary pairs was determined along each line. From the 149 pairs observed, 17% depicted countercurrent flow and 83% concurrent flow, amounting to a concurren t/countercurrent ratio of 4.96.

Despite the elegance of these intravital approaches, the intrinsic contractile nature of cardiac activity unfortunately presents formidable technical difficulties for a reliable quantitative determination of capillary flow pattern. As a result, direct visualization of coronary capillaries by means of intravital microscopy, whether by epi- or transillumination of the myocardial wall, has yielded only limited qualitative data regarding flow direction. These techniques require manipulation of the tissue which in itself may alter the flow pattern of the region

under study. In addition, they are limited to the superficial layers of the epimyocardium, which may not be representative of the deeper layers of the myocardium whose environment and thus flow is affected to a greater degree by functional changes arising from, for example, extravascular coronary resistance, fluctuations in contractile state, as well as vasomotor tone.

Aside from theoretical estimates of coronary capillary flow direction based on geometric modelling, actual descriptions of transmural flow pattern have, to date, proven elusive. In an effort to resolve the inconsistencies of previous results as well as to provide a reliable means for transmural analysis of myocardial flow direction, it was crucial to develop a new approach to this problem. To this end, Reeves and Rakusan (1988) described a new technique which utilizes the capability of non-radioactive colored microspheres to produce capillary "flow vectors". This technique, by providing a permanent record of microvascular flow activity during the period of microsphere infusion allows for quantitative information to be obtained concerning myocardial capillary flow direction in a variety of experimental environments.

STATEMENT OF THE PROBLEM 2

Despite the importance of delineating a specific microvascular flow pattern adapted to the functioning of the heart, under both normal and pathologic conditions, this objective has proven particularly difficult in view of the cyclic nature of cardiac activity. In fact, until the application of the colored microsphere method as introduced in our laboratory, no technique had emerged which would allow for the accurate analysis of this parameter throughout the whole heart. In this context, the direction of blood flow in neighboring coronary capillaries i.e., concurrent vs countercurrent flow, is regarded as a potentially significant determinant of tissue oxygenation. Given the shortcomings of previous investigations in this area, the specific direction of this portion of the dissertation will be to address the following questions:

1. Utilizing a novel technique incorporating sequential *in vivo* infusion of differently colored non-radioactive microspheres into rat myocardium, could regional capillary flow direction be determined quantitatively in the left ventricular free wall?

2. Since the potential imbalance between oxygen supply and oxygen demand can be exacerbated in hearts hypertrophied by a pressure overload, would the overloaded myocardium exhibit alterations in regional capillary flow pattern in an effort to sustain normal tissue oxygenation?

3. Could an in vitro model using the isolated perfused heart yield reliable data regarding capillary flow direction? If so, would the technical advantages of an in vitro model provide a satisfactory method for determining capillary flow direction in the developing myocardium? Given that the capillary network in the developing heart exhibits systematic differences in geometry relative to the adult heart, would such changes with growth be reflected by adaptations in capillary flow direction?

4. Finally, would the technical conveniences and superiority of an in vitro model for inducing an acute hypoxic state be suitable for an evaluation of potential alterations in the normal capillary flow pattern, due to a lowered arterial oxygen tension ?

CHAPTER 3: MICROVASCULAR FLOW VECTORS IN NORMAL AND HYPERTROPHIC RAT MYOCARDIUM

INTRODUCTION

It is the function of the coronary capillaries to provide tissue oxygenation for the requirements of mechanical function, and to respond rapidly to changing oxygen demands. These demands are met partially by flow adjustments such as changes in cardiac output and systemic blood pressure, or in the tone of arteriolar smooth muscle. In addition, coronary microvascular architecture can play an important role in oxygen supply by influencing the spatial distribution of these oxygen sources, i.e. the myocardial capillaries. Analysis of transport mechanisms must take into account not only blood flow through individual microvessels, but also the influence of capillary arrangements on tissue oxygenation (Lübbers, 1976; Grunewald and Sowa, 1978). In this connection, the direction of blood flow in neighboring capillaries can be of importance i.e. the presence of concurrent or countercurrent flow (Diemer, 1965).

As described in the introduction to this portion of the dissertation, intravital techniques require an undue degree of manipulation of the heart which may in fact alter the existing regional flow pattern. In addition, observations are typically limited to the surface layers of the heart, which is not representative of the deeper and more vulnerable regions such as the subendocardium. Moreover, the local contractile state and high tissue pressures generated may significantly affect

subendocardial blood flow. The precarious balance between oxygen delivery and oxygen demand in comparison to the epi- and midmyocardium may render it more susceptible to conditions of critical oxygen supply (Griggs et al., 1973; Eng et al., 1987). This potential imbalance can be exacerbated in hearts hypertrophied by a pressure overload, where a decreased capillary density and an increased variability in capillary spacing is typically observed (as reviewed by Rakusan, 1987).

In an effort to unravel the incongruities arising from earlier *in vivo* microscopy studies, as well as to provide a method for regional analysis of microvascular flow direction, a novel technique was developed incorporating sequential in vivo infusion of differently colored non-radioactive microspheres into rat myocardium (Reeves and Rakusan, 1988). The purpose of the present study was to investigate capillary flow direction in both normal and hypertrophied rat myocardium, and consider what regional adaptations, if any, occur with respect to flow direction.

METHODS AND MATERIALS

Experimental animal preparation:

Sixteen male Sprague-Dawley rats were randomly divided into two groups: pressure overload hypertrophy (n=7) and their sham-operated matched controls (n=9). Cardiac hypertrophy was produced by aortic constriction in six-day-old neonatal rats. While the animals were under sustained ether anesthesia, a midline abdominal incision was performed, with the intestinal tract reflected to one side to

allow access to the abdominal aorta. A sub-diaphragmatic suprarenal ligature was tied around the aorta, with a 30-gauge hypodermic needle serving as the template. The animals were allowed to recover, and at 6 to 7 weeks postsurgery, were placed in a flow through system and anesthetized with 2.5% halothane in oxygen, delivered at 500 ml/min.

After surgical preparation, a midline incision was made from the cricoid cartilage to the sternal notch. Blunt dissection and subsequent retraction of strap muscles exposed the carotid arteries and trachea. With the limbs extended and restrained, a radiopaque teflon catheter was inserted into the trachea through a tracheostomy and secured with a 4-0 silk ligature. The animals were then connected to a rodent ventilator (Harvard) delivering 1.5% halothane in oxygen at 500 ml/min. The left carotid artery was catheterized with a PE-50 polyethylene cannula for the continuous recording of heart rate (ECG) and mean arterial pressure from a Grass Model 7 Polygraph. Following palpation, the left atrium was exposed by a small lateral thoracotomy and gently supported with forceps. A bevel-tipped cannula (PE-10), stabilized by a metal stylet, was inserted into the left atrial appendage by puncture. The metal stylet was immediately removed, and the cannula flushed with 0.2 ml heparinized saline (12.5 mg%).

Colored microsphere procedure:

Thereafter, two sequential injections of differently colored 1 ml microsphere aliquots were carried out, each containing approximately 10 million, 10 ± 0.2 μm microspheres (obtained from EZ-TRAC, Los Angeles, CA) suspended in .01%

Tween solution. The combination of this surfactant along with vigorous vortexing minimized any microsphere aggregation prior to injection. Infusions were conducted over a two-minute period and quickly followed by a 0.5 ml saline flush. A five-minute interval elapsed between injections.

After all injections were concluded, the hearts were excised, weighed, dissected, and frozen in liquid nitrogen for subsequent cryotomic preparation of 40 μm longitudinal tissue sections from both midmyocardial and subendocardial regions. The sections were then fixed (5% acetic acid, 95% absolute alcohol) for 1 minute and stained with the Periodic Acid-Schiff method. All tissue samples were coded, with the code being revealed only at the time of data compilation and analysis.

The fundamental rationale behind the colored microsphere technique for the characterization of capillary flow direction is based on the finding that sphere movement into a vessel of smaller resting diameter occurs through circumferential distention of the capillary wall. Further progression through a capillary remains possible until the intraluminal driving pressure becomes insufficient to overcome inherent wall tension, and/or a limiting vascular diameter results in sphere lodgement or entrapment. This phenomenon has been observed in the microcirculation of rat gracilis muscle. Intravital microscopy revealed the entry of 10 μm microspheres into capillaries with subsequent intracapillary aggregate formation. In addition, arterially injected dye flowed past trapped microspheres as did an occasional red blood cell (Reeves et al., 1989).

The procedure, as applied in the present investigation resulted in the formation of 3 distinct microsphere aggregation patterns within the coronary microvasculature.

1) single sphere

2) aggregates of one color of microsphere

3) aggregates containing both colors of microspheres

Detailed microscopy revealed that numerous capillaries contained sphere aggregates of both colors. According to aggregation patterns, a "flow vector" was established based on the sequence of microsphere colors entrapped within each capillary. In this manner, an examination of "flow vectors" obtained from neighboring capillaries provided the information necessary to characterize the direction of flow existing between capillaries during the period of microsphere infusion (Plate 4a and 4b). For the present study, this analysis was expanded to incorporate a "capillary ranking", each increasingly removed from an individual "reference" capillary.

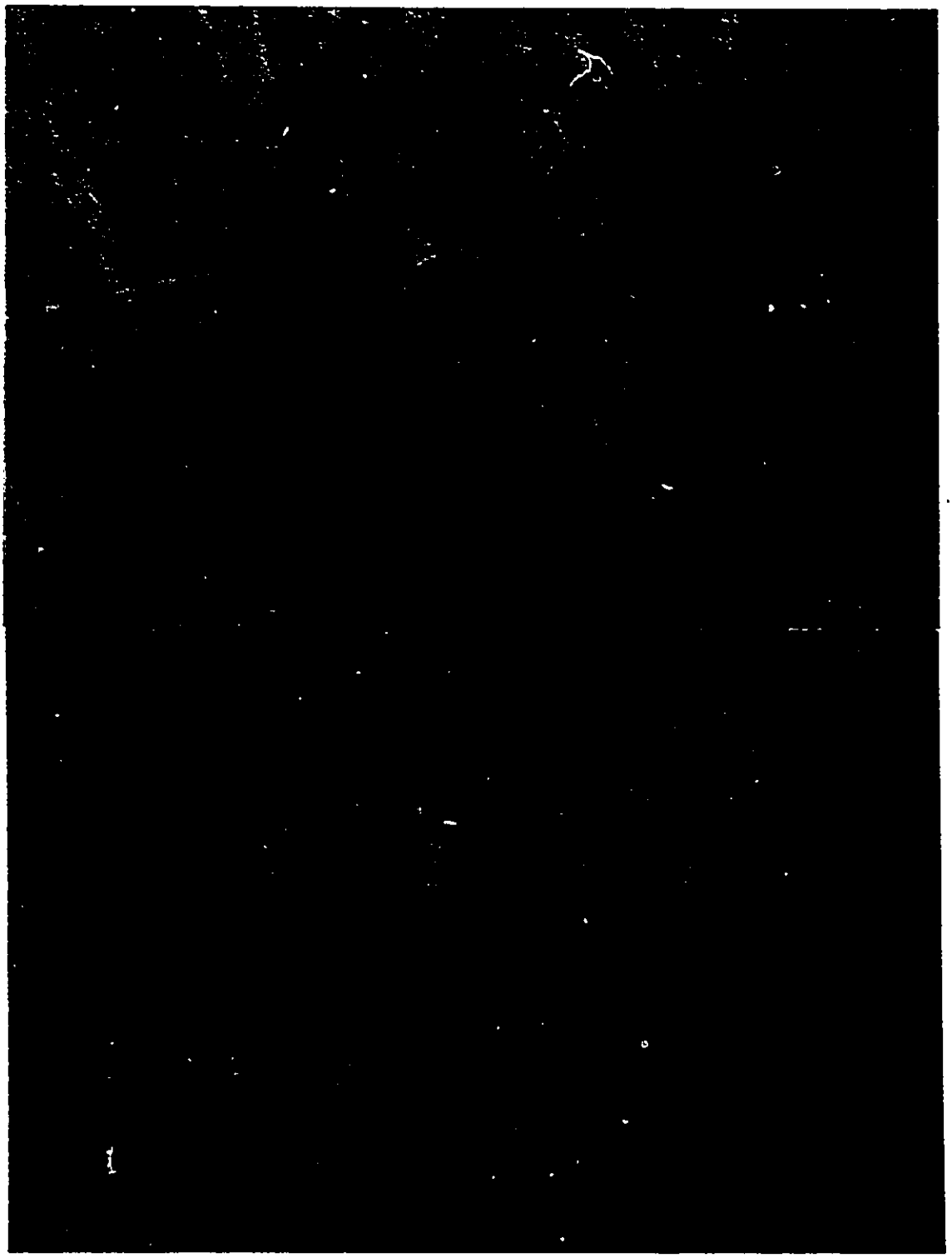


Plate 4 Photographic representation of capillary "flow vectors" in plates **A** and **B** based on the sequence of differently colored microspheres trapped within neighboring coronary capillaries. Arrows indicate flow direction. Mag. 500x

TABLE 1.

Body weight, left ventricle weight, and hemodynamic data in control (CON) and hypertrophic (HYP) rat myocardium recorded at the time of colored microsphere infusions.

	CON (n=9)	**HYP (n=7)**
Body Weight (g)	263 ± 4	237 ± 8
Left ventricle (mg)	586 ± 10	976 ± 57*
LV/BW ratio (mg/g)	2.2 ± 0.3	4.1 ± 0.3*
Heart rate (bpm)	358 ± 13	422 ± 8*
MAP (mmHg)	107 ± 6	167 ± 15*

Mean ± SEM *= sig. diff. from controls (Student's t-test; $p<0.05$)

TABLE 2.

Flow vector observations with corresponding percentages of concurrent flow recorded in control (CON) and hypertrophic (HYP) hearts.

CAPILLARY RANKING:	1	2	3	4	5
CON (n=9)					
Total Observations (MID)	390	524	510	492	422
% Concurrent	86%	84%	82%	79%	77%
Total Observations (SUB-E)	326	478	386	370	314
% Concurrent	72%	61%	64%	56%	52%
HYP (n=7)					
Total Observations (MID)	326	382	418	388	320
% Concurrent	88%	81%	84%	80%	70%
Total Observations (SUB-E)	372	404	430	328	308
% Concurrent	67%	58%	57%	62%	55%

Note: Each level of capillary ranking describes the sequence of positions increasingy removed from an individual reference capillary. Both groups are further subdivided according to the regional subdivisions of MID and SUB-E

RESULTS

Mean arterial blood pressure was found to be significantly greater in the hypertrophic hearts than in their control counterparts ($p<0.05$). The slow onset model of pressure overload utilized in the investigation resulted in a significant increase in cardiac mass (Table 1). Left ventricular weight increased 67% in hypertrophied hearts relative to sham operated controls. Left ventricular to body weight ratio's increased by 78%. A slight, though statistically significant tachycardia was also found to be present in the hypertrophic group.

The flow vector observations in both control and experimental groups for the regional subdivisions according to capillary ranking are summarized in Table 2. To verify reproducibility in flow vector determination, test-retest comparisons on 20 randomly selected tissue specimens were conducted over the intercapillary range. A difference of less than 7% was obtained, indicating a sufficient measure of reproducibility in observation and recording technique throughout the investigation.

In general, these observations reflected a steady decrease in percentage of concurrent flow in both control and experimental groups as the ranking position from an individual reference capillary increased. Additionally, regional differences in both control and experimental groups were present, with subendocardial regions demonstrating lower levels of concurrent flow relative to midmyocardium at each level of capillary ranking.

A 3-way nested factorial ANOVA for proportions (percentages) utilizing an arc sin transformation of the dependent variable data (Armitage and Berry, 1987)

was the procedure of choice to investigate the contributions and statistical significance for independent variables of capillary ranking, control-hypertrophy (CON-HYP), midmyocardium/subendocardium (MID/SUB-E), with respect to the flow vector observations. The individual hearts were treated as a nested factor within the grouping variable of (CON-HYP). These results are illustrated graphically in Figures 11 and 12. Figure 11 combines the observations over the range of capillary rankings and reveals that global differences with respect to flow direction between CON and HYP hearts were not evident. In addition, both groups demonstrated the same statistically significant decline ($p<0.001$) in percentage of concurrent flow from MID to SUB-E. In Figure 12, the results from control and experimental groups have been combined (due to lack of siginificant diferences) to illustrate the influence of capillary ranking on overall regional differences. The subendocardial regions depicted significantly lower percentages of concurrent flow relative to midmyocardial regions, with a decline in the percentage of concurrent flow as the ranking position from a reference capillary increased.

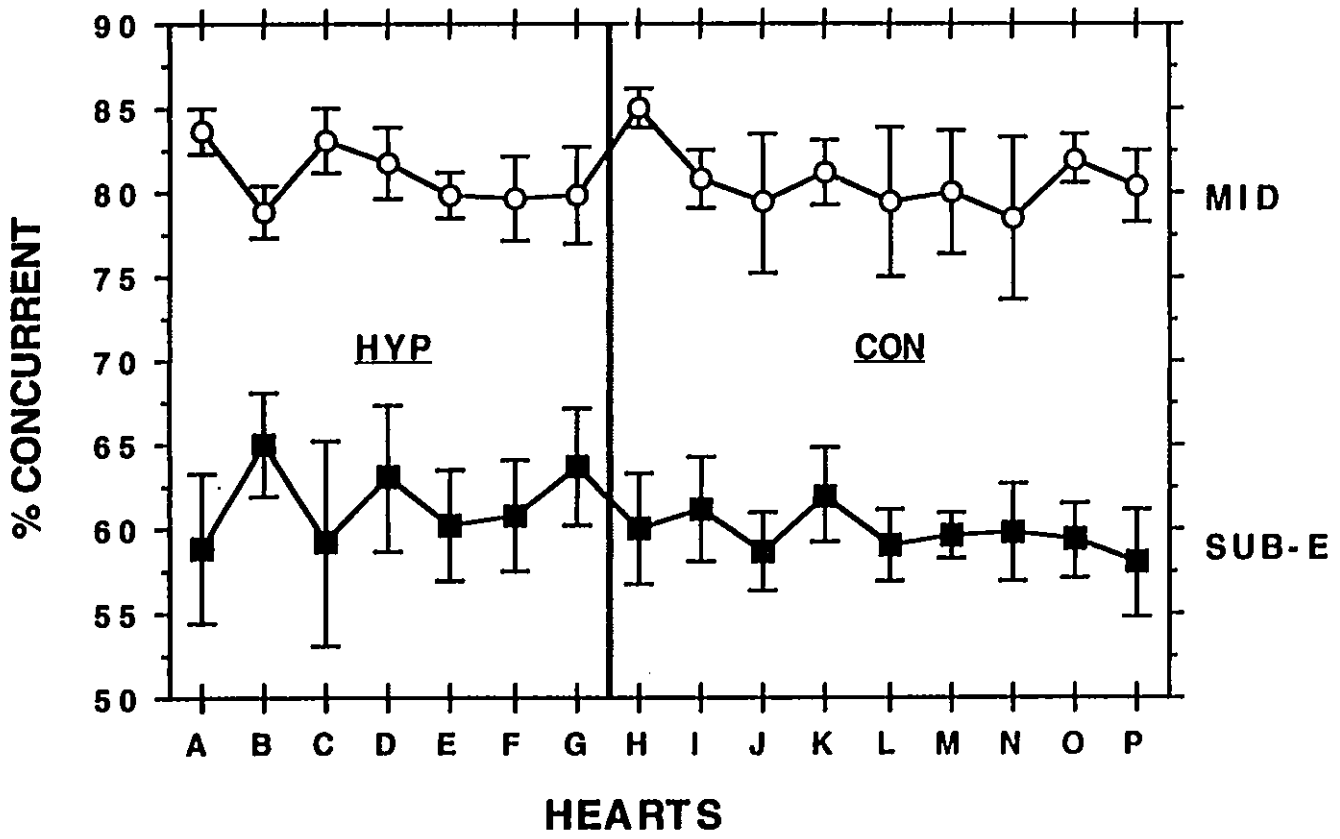


Figure 11. Graphical representation of the percentage of concurrent flow combined over the range of capillary rankings for HYP hearts (A to G) and CON hearts (H to P). ANOVA did not detect statistically significant differences between HYP and CON groups. However, regional subdivisions (MID vs SUB-E) in both HYP and CON were significantly different ($p<.001$). Variability within each heart, as depicted by standard errror bars also illustrated.

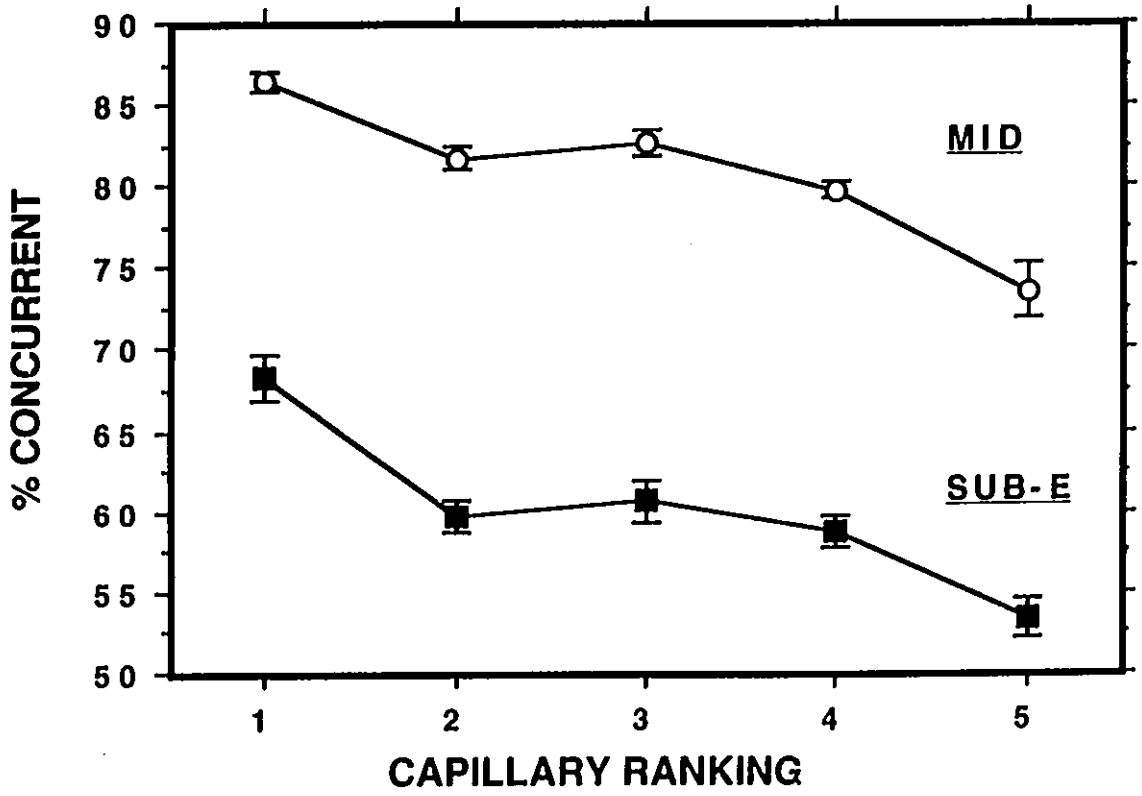


Figure 12. The effect of capillary ranking on percentage of concurrent flow is illustrated for MID and SUB-E regions. In both cases the overall effect of increasing capillary ranking on percentage concurrent flow was statistically significant ($p<.001$). HYP and CON values have been combined due to lack of significant differences. Points represent means ± SEM.

DISCUSSION

The colored microsphere technique as applied in the present study provided a permanent record of flow activity during the period of microsphere infusion. In this manner, both qualitative as well as quantitative knowledge regarding transmural capillary flow direction has been obtained for the first time in normal myocardium, and in experimental cardiomegaly.

To determine the nature of capillary flow direction in myocardium requires the presence of "flow vectors" among neighboring capillaries. For the purposes of the present study this necessitated infusing approximately twenty million microspheres through the left atrial appendage to establish the baseline flow pattern. While such numbers may satisfy the requirement of attaining an adequate number of flow vector observations, it would appear to present two possible limitations to the method itself: (1) the injection of such a large quantity of microspheres into the microcirculation may alter hemodynamics and thus flow patterns. (2) the presence of a microsphere aggregate in one capillary may alter local flow, especially in closely neighbouring capillaries.

With respect to these methodological concerns some simple mathematical analysis may prove useful. If we consider that an average capillary density (the number of capillaries found in a tissue cross-section perpendicular to the direction of the main capillaries) in adult rat myocardium is approximately 3000/mm^2 (i.e., 6000 capillaries/mm^3 of tissue. assuming an average capillary length of approximately 0.5 mm) (Ludwig, 1971; Rakusan, 1971), then even with approximately

800,000 microspheres entering the coronary microvasculature, only about 5% of the capillaries in any given cross section would be expected to contain microspheres. Since numerous vessels trap more than one microsphere, the number of capillaries obstructed would be considerably lower than 5%. Thus, with less than 5% of coronary capillaries containing microspheres, one would predict only minor alterations in coronary flow and left ventricular function. This was borne out during the series of experiments. Hemodynamic parameters remained essentially constant during the periods of infusion.

When considering the effects of microsphere lodging on local flow pattern one might be initially surprised to see that 10 μm microspheres can enter capillaries having smaller resting diameters than the microspheres themselves. Furthermore, microsphere lodging is often found to be sequential, as reflected by the presence of multicolored aggregates. This suggests two important points: 1) the capillary wall is not rigid, but rather may be capable of considerable dilatation, and 2) flow within a microsphere containing capillary is still maintained, thus minimizing the effect on flow pattern in neighbouring capillaries.

The effect of microsphere lodging has been investigated through *in vivo* videomicroscopic observation of microsphere movement in the microcirculation of rat gracilis muscle (Reeves et al. 1989). Detailed observations revealed the entry of 10 μm microspheres into capillaries through circumferential distention of capillary walls. The investigators also observed the subsequent formation of intracapillary microsphere aggregates. Furthermore, arterially injected dye flowed past trapped microspheres, as did an occasional red blood cell. These observations

provided evidence against the argument that aggregate formation was more likely to occur upstream, and thus enter the capillary network as "preformed" aggregates. Since the microspheres were not tightly occlusive, other microspheres could still enter capillaries and form aggregates.

One noteworthy finding in the present investigation was the high overall percentage (87%) of concurrent blood flow between adjacent capillaries. This is in general agreement with our previous results in rat midmyocardium (Reeves and Rakusan, 1988), as well as results derived from *in vivo* transillumination of canine myocardium (Tillmans et al., 1974), and *in vivo* epi-illumination of skeletal muscle capillary beds (Tyml et al., 1981).

It has previously been described that transverse arterioles cross the supplied capillary beds at oblique angles, with the origins of capillaries often found emanating from a given transverse arteriole in a staggered array (Brown, 1965; Ludwig, 1971; Rakusan, 1971; Grote and Thews, 1973; Reeves and Rakusan, 1988). This notion of staggered terminal arteriolar inflows and venular outflows relative to the supplied capillary beds was described in greater detail by Batra et al. (1989). Their model further supported the idea that the arteriolar and venular portions of capillaries would not be in register with one another. Staggering of capillary origins could have a direct effect on the transport of oxygen to the surrounding tissue, since neighboring capillaries are no longer at equivalent oxygen tensions, as would be predicted for purely symmetric concurrent capillary origins. This distinctive microvascular geometry could theoretically overcome a disadvantage of concurrent flow between adjacent capillaries by ensuring that

arterial PO_2 is more efficiently utilized, thereby providing adequate oxygen delivery and nutrient support to local cardiac myocytes.

The high percentage of concurrent flow was essentially preserved in pressure overload hypertrophy. Indeed, pressure overloaded hearts did not exhibit any significant differences relative to their normal sham-operated counterparts. It has been well established that when pressure overloading is induced in younger animals, greater compensatory capillary proliferation occurs compared to pressure overloading in adult animals (see Rakusan, 1987). One important aim of the study was to maximize the hypertrophic response in myocardium, consistent with the large increases in cardiac mass associated with cardiovascular pathologies such as severe systemic hypertension or aortic stenosis. In this connection, it has previously been demonstrated that introduction of a pressure overload in neonatal animals results in a far greater increase in cardiac mass in comparison to pressure overload incorporating a similar degree of aortic constriction in adult animals (Rakusan and Korecky, 1985; Campbell et al., 1989). It is nevertheless possible that our results may, at least in part, be due to imposition of aortic constriction in these young animals. Whether similar capillary flow patterns would prevail with cardiac hypertrophy induced in adult or aged animals is unknown. Our findings, therefore, should be interpreted solely with respect to the present slow-onset model of aortic constriction in neonatal rats.

The finding that the percentage of concurrent blood flow decreased significantly among capillaries further removed from an individual reference capillary

increased may reflect the presence of distinct arteriolar units (see Skalak and Schmid-Schönbein,1986). This concept is further supported by a previous study of capillary beds in hamster tibialis anterior muscle (Lund et al., 1987). Epifluorescence videomicroscopy, in concert with fluorescein-stained albumin revealed, that capillaries originating from a given arteriole travelled in parallel, with essentially concurrent flow direction within a given microvascular unit. The majority of countercurrent flow observations resulted from neighboring capillaries found to be emanating from separate microvascular units with independent internal flow patterns. Similar flow strategies conceivably exist in myocardium, i.e., predominantly unidirectional flow among neighboring capillaries within a given unit, while a bordering (microvascular) arteriolar unit possessing its own discrete flow arrangement, might be oriented in countercurrent fashion to the previous unit. Therefore, the further removed from an individual reference capillary that a flow vector was recorded, the greater the likelihood of capturing a capillary belonging to a separate arteriolar unit.

Significant regional differences were evident in both control and hypertrophic hearts. The overall percentage of concurrent blood flow was significantly lower in the subendocardial regions in both groups. While no ready explanation for these observations is available from the data presented, several interesting possibilities may shed light on these occurrences. The architecture and distribution of the intramyocardial arteriolar network are still largely unknown. It can be postulated however, that the myocardial arteriolar unit narrows transmurally, as the circumferential dimensions of the heart decrease towards the endocardial

surface. This would have a tendency to space adjacent units closer together, with the greater likelihood of encountering countercurrent flow observations if individual arteriolar units were to display a discrete internal flow pattern. Although there is a relative paucity of quantitative data on arteriolar architecture in the rat heart (Rakusan and Wicker, 1990), several recent reports have indicated a greater arteriolar vascular density and/or resistance distribution in the subendocardium relative to the subepicardium (L'Abbate et al., 1980; Izumi et al., 1984; Pelosi et al., 1990). On the other hand, studies on regional differences in capillary density report conflicting results. While there are reports of lowered capillary densities in the subendocardium of dogs (Gerdes and Kasten, 1980), pigs (White et al., 1987), and in rats exposed to experimental volume overloaded hypertrophy (Rakusan et al., 1980), other investigations have not documented significant regional differences (eg. Anversa et al., 1978; Vetterlein et al., 1982; Poole and Mathieu-Costello, 1990).

If indeed arteriolar vascular density is greater in the subendocardium, with a relative constancy in regional capillary density, it would not seem unreasonable to envisage the arteriolar/capillary ratio as being elevated in the subendocardium. This further points to the possibility of neighboring arteriolar units with their respective and smaller capillary bundles being in closer proximity to one another. Therefore, capillaries further removed from an individual reference capillary could conceivably belong to a separate arteriolar unit. Finally, reflection of a myocardial arterial unit from the endocardial surface could create a characteristic U turn

geometric orientation, with an apparent countercurrent flow pattern among capillaries within the unit.

In the present chapter, we have demonstrated to the best of our knowledge the first reliable quantitative determinations of regional capillary flow direction in the mammalian heart. The advent of colored microsphere technology enabled us to devise a strategy in describing this phenomenon in the previously inaccessible mid- and subendocardial regions of the left ventricle. The foregoing results also represent the first reliable characterizations of flow direction in both normal and pathophysiologic states. Such data, in conjunction with morphometric descriptions of capillary geometry, are essential as input parameters for the precise modelling of myocardial oxygen transport, toward the goal of a detailed understanding of blood-tissue gas exchange.

CHAPTER 4: THE EFFECT OF AGE ON CAPILLARY FLOW DIRECTION IN THE ISOLATED PERFUSED RAT HEART

INTRODUCTION

In Chapter 3 we described coronary capillary flow direction with respect to regional differences in normal hearts, as well as hearts subjected to pressure overload hypertrophy utilizing our novel colored microsphere technique.

The first objective of the present investigation was to determine whether the isolated perfused heart according to Langendorff was a suitable model for the determination of capillary flow vectors using our colored microsphere technique. The isolated perfused heart according to Langendorff remains, 95 years after its first description, one of the most popular and useful techniques available for studying the hemodynamics, metabolism, and histological structure of the heart. Nevertheless, the Langendorff preparation has been criticized periodically because it is lacking a normal neural and humoral background, and therefore does not function mechanically as would the intact heart *in vivo*. Consequently, experimental results obtained from this model are often regarded as somewhat " non-physiological". Such potential shortcomings must be taken into account when proposing an investigation. On the other hand, the comparative ease of the technical set-up and surgical preparation, with the ability to precisely control various parameters, should not be undervalued. Ultimately, the ability of the isolated heart to provide potentially useful information required an evaluation on

the basis of results from previously worked out standards. For the present study, these standards for comparison were provided from our earlier *in vivo* study of capillary flow vectors in myocardium.

A second important objective which is presented in this chapter is an examination of capillary flow vectors during early growth and development of rat myocardium. The developmental period is, in fact, characterized by structural integration of numerous time-dependent phenomena. Prior to birth, increases in cardiac mass are distinguished primarily by myocyte hyperplasia, ie., an increased number of myocytes. Thereafter, extending through the weaning period there is a gradual shift to hypertrophic myocyte growth (Anversa et al., 1980; Rakusan, 1984). Following this stage, further increases in cardiac mass are the result of myocyte hypertrophy, ie., an increased size of the individual myocytes without changes in their total numbers (Rakusan et al., 1965). Coupled with this increase in cardiac mass is found a progressive growth and expansion of the coronary microvascular network (Rakusan, 1984). During the early postnatal period, numerical capillary growth surpasses that of the myocytes (Anversa et al., 1986), and the capillary/fiber ratio increases several fold (Rakusan and Poupa, 1963; Rakusan et al., 1965; Olivetti et al., 1980). During the first 11 days of postnatal development in the rat, there are 2 to 3-fold increases in the ratios of capillary volume and surface, to myocyte volume and surface, respectively (Olivetti et al., 1980). The increasing capillary proliferation during the early postnatal period leads to maximal capillary density at approximately 3 weeks of age in the rat heart (Rakusan and Poupa, 1963; Rakusan et al., 1965). These changes are believed

related to the increased oxygen requirements of the developing rat heart. Employing discriminating histochemical and morphometric methods, Batra and Rakusan (1992) identified systematic differences in cross-sectional and longitudinal capillary parameters during the post-weaning period of cardiac development in the rat. These differences included a pronounced increase in the number of parallel running capillaries comprising individual capillary sets with growth.

Therefore, if our initial objective was successful in establishing the applicability of the colored microsphere technique in an *in vitro* model, then it was considered feasible to apply it towards the characterization of capillary flow vectors in developing myocardium. The potential merit of this approach over *in vivo* infusion was of particular relevance in the case of younger hearts, which present considerable technical problems when assessed through *in vivo* techniques. Accordingly, we decided to characterize capillary flow vectors in the developing heart relative to the adult heart using the isolated perfused Langendorff model, in order to determine if the reported changes in the capillary net with growth were reflected by similar changes in capillary flow direction.

METHODS AND MATERIALS

Experimental animal preparation:

To investigate regional capillary flow direction in rat myocardium an isolated perfused "Langendorff" model was utilized for the sequential infusion of differently colored microsphere suspensions. Sprague-Dawley rats 6-7 week old

(n=10), as well as 3-week old (n=34) were anesthetized with sodium pentobarbital (0.1ml/100g body weight). A midline laparotomy was performed with the inferior vena cava isolated. Heparin (0.1ml/100 g body weight) was then injected and allowed to circulate for one minute. Subsequently, the abdominal aorta and inferior vena cava were severed to bleed the animal. Once breathing had ceased, the heart was exposed and loosely packed with crushed ice to arrest it. The aorta was then isolated with a small incision made below the branch of the right carotid artery. A stainless steel canula primed with saline (containing 0.5 mM $CaCl_2$) was inserted into the aorta with 4/0 silk suture tightened around the aorta and cannula. The heart was quickly excised and the cannula was attached to a Langendorff perfusion apparatus. Hearts were perfused at a constant pressure of 80 cm of H_2O in adult and 65 cm of H_2O in 3-week old hearts respectively. These levels were chosen to reflect the in vivo difference in arterial blood pressure in both age groups. The Krebs-Henseleit bicarbonate perfusate contained ($mmol \cdot L^{-1}$) : NaCl 118.0; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; glucose 7.0; sodium pyruvate 2.0, and mannitol 1.1. The perfusate was equilibrated at pH 7.4 with 95%O_2-5%CO_2 and warmed to 37°C in a water-jacketed column.

A compliant balloon-tipped catheter was placed in the left ventricle (deflated) and held in place by the mitral valve. A small, known volume of water was added to the balloon in order to produce an end-diastolic pressure of approximately 6 mmHg. The peripheral end of the catheter was attached to a transducer (Statham Db 23) and recorder (Grass Model 7) to monitor left

ventricular developed pressure, and dP/dt continuously. The hearts were electrically stimulated at a constant rate of 300 beats/minute using small, silver, spiral electrodes connected to the base of the right ventricle. Coronary flow rate (effluent from the right ventricle) was measured using a graduated cylinder for a thirty second period.

Colored microsphere procedure:

On the basis of our earlier *in vivo* experiments which indicated minimal hemodynamic alterations when infusing microspheres with diameters of 10 ± 0.23 μm, we decided to utilize this diameter of microsphere for the present in vitro experiments. Prior to injection, the microspheres were suspended at a concentration of 6×10^6/ml in saline containing .01% Tween solution, and lightly agitated in a vortex mixer for one minute. Microsphere aggregates were not observed during microscopic examination of the suspension. Thereafter, differently colored 2 ml microsphere aliquots, each containing approximately 6×10^5 red or blue microspheres, were infused sequentially into the ascending aorta (retrograde perfusion through the coronary vasculature) at constant and identical rates of 0.2 ml/min for 10 minutes (Adult) or 3 minutes (3-week-old) via a Sage Instruments Model 355 syringe pump. A 5-minute interval elapsed between injections. After all injections were concluded, the hearts were weighed, dissected, and the samples quick frozen for cryotomic preparation of 40μm longitudinal sections from midmyocardial and subendocardial regions.

The rationale behind the use of the colored microsphere technique for the characterization of capillary flow direction has been described in Chapter 3. The sequential infusion of two differently colored microsphere suspensions results in the formation of three distinct aggregation patterns within the coronary microcirculation. According to aggregation patterns, a "flow vector" was established based on the sequence of microsphere colors entrapped within each capillary. Detailed microscopic examination of flow vectors obtained from neighboring capillaries enabled the characterization of regional capillary flow direction.

RESULTS

Body weight and left ventricular weight data from the two series of *in vitro* experiments comprising the present study are shown in Table 3. The adult group was characterized by a 6.6-fold and 4.7-fold increase in body weight and left ventricular weight respectively in comparison to the 3-week old animals. As a result of a substantially smaller cardiac mass in the 3-week old animals, a greater number of hearts (n=34 vs n=10) was required for sampling in order to achieve comparable flow vector observation numbers. Table 4 illustrates a comparison of the percentages of concurrent flow vector observations from our previous *in vivo* study to the present isolated heart experiments comprising adult and 3-week old hearts.

TABLE 3.

Body weight (BW), Left ventricle weight (LV), and left ventricle/body weight ratios (LV/BW) in YOUNG (21-day-old) and ADULT (6-week-old rats)

	YOUNG (21-DAY)	ADULT (6-WEEK)
Body weight (g)	39 ± 2	256 ± 4 *
Left ventricle (mg)	127 ± 4	594 ± 8 *
LV/BW ratio (mg/g)	3.3 ± 0.1	2.3 ± 0.3 *

Mean ± SEM *= sig. diff. (Student's t-test; $p < 0.01$)

TABLE 4.

Percentages of concurrent flow according to the sequence of capillary rankings for IN VIVO ADULT and IN VITRO (ADULT and 3-WEEK-OLD) groups. Each group further subdivided according to MID and SUB-E regions.

CAPILLARY RANKING:	1	2	3	4	5
IN VIVO ADULT (n=9)					
% Concurrent (MID)	86%	84%	82%	79%	77%
% Concurrent (SUB-E)	72%	61%	64%	56%	52%
IN VITRO ADULT (n=10)					
% Concurrent (MID)	87%	82%	81%	78%	75%
% Concurrent (SUB-E)	79%	74%	63%	65%	58%
IN VITRO 3-WEEK (n=34)					
% Concurrent (MID)	83%	78%	73%	75%	70%
% Concurrent (SUB-E)	80%	74%	66%	69%	63%

To investigate possible differences between the *in vivo* results presented in Chapter 3 and the present isolated heart data derived from adult rats, a three-way nested ANOVA for proportions was selected to examine the contributions and potential significance for the independent variables of capillary ranking, in vivo versus in vitro, midmyocardium versus subendocardium (MID/SUB-E), and the individual hearts to the flow vector observations. In both experimental models, results indicate a predominance in concurrent flow direction, which decreased ($p < 0.001$) with capillaries increasingly removed from an individual reference vessel.

A regional comparison between data derived from our previous *in vivo* experiments and data from the present isolated heart (ADULT) group is illustrated in Figure 13. No statistically significant differences were revealed in midmyocardium. With respect to subendocardial regions, significant differences were revealed ($p < 0.05$), with higher percentages of concurrent flow present in the isolated heart group relative to the *in vivo* group. Additionally, within both groups, there was a significant difference revealed ($p < 0.01$) in percentage of concurrent flow between mid- and subendocardium.

The same statistical model was utilized to test for possible differences between the (ADULT) isolated heart group vs the (3-Week-old) group. The 3-week old group also revealed that the percentage of concurrent flow declined significantly ($p < 0.001$) with capillaries further removed from a reference vessel (Figure 14). Notably, the effect of capillary ranking was not significantly different between the two groups. Figure 15 illustrates a regional comparison between the isolated heart group (ADULT) and (3-Week-old) group. Statistically significant

regional differences within both groups were found ($p < 0.05$), with higher percentages of concurrent capillary flow in midmyocardium relative to subendocardium. However, when comparing percentages of concurrent flow in either midmyocardium or subendocardium between the adult and 3-week-old groups, no statistically significant differences were revealed. The above results suggest that age was not an important factor with respect to capillary flow direction in the isolated perfused rat myocardium.

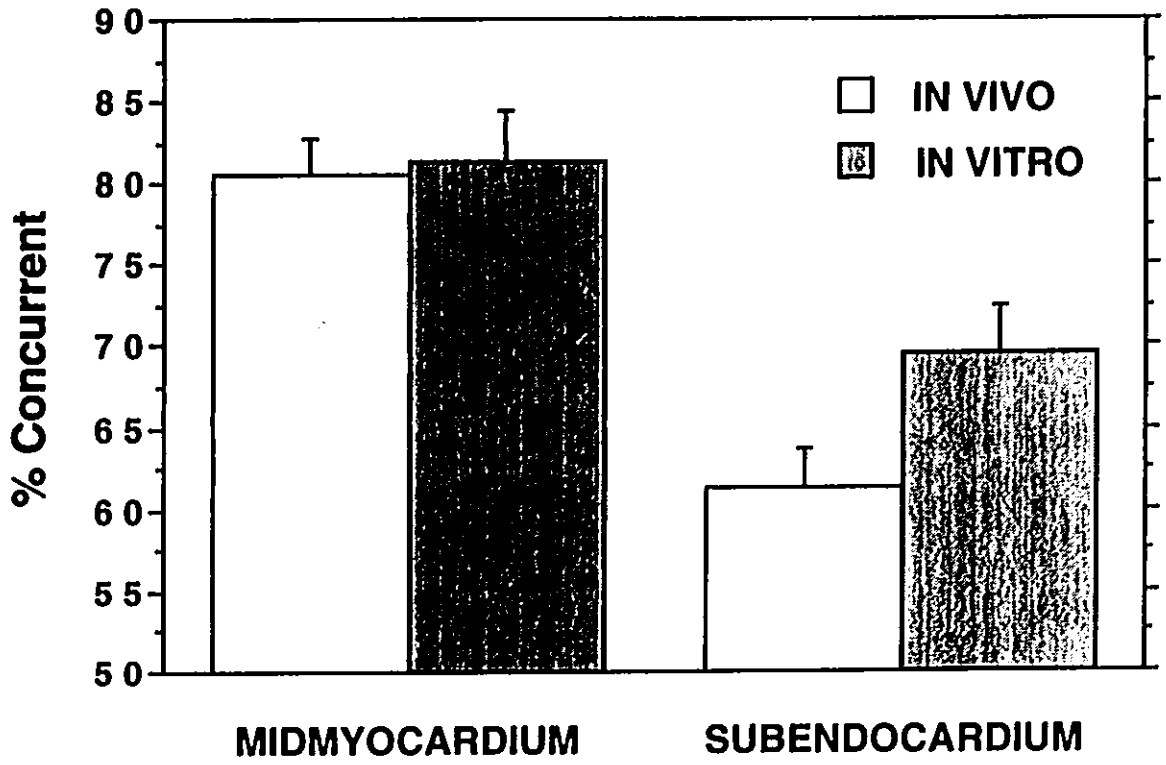


Figure 13. Percentages of concurrent flow for IN VIVO and IN VITRO (ADULT) groups in midyocardial and subendocardial regions. Both groups revealed higher percentages of concurrent flow in midmyocardium ($p<0.01$). The IN VITRO group depicted higher percentages of concurrent flow in subendocardium compared to the IN VIVO group ($p<0.05$). Bars represent means ± SEM.

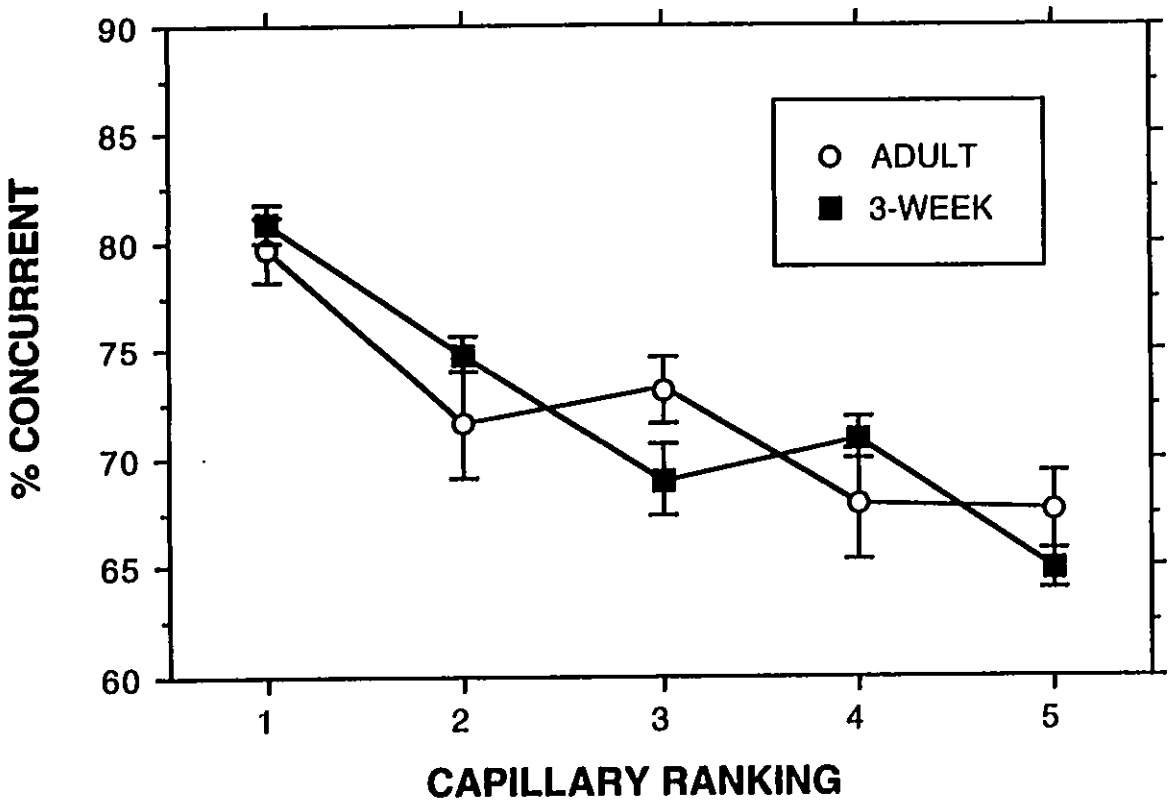


Figure 14. Graphical representation of the effect of capillary ranking on the percentages of concurrent flow in isolated ADULT and 3-WEEK old hearts. In both groups the overall effect of capillary ranking was significant ($p<0.01$). Points represent means ± SEM.

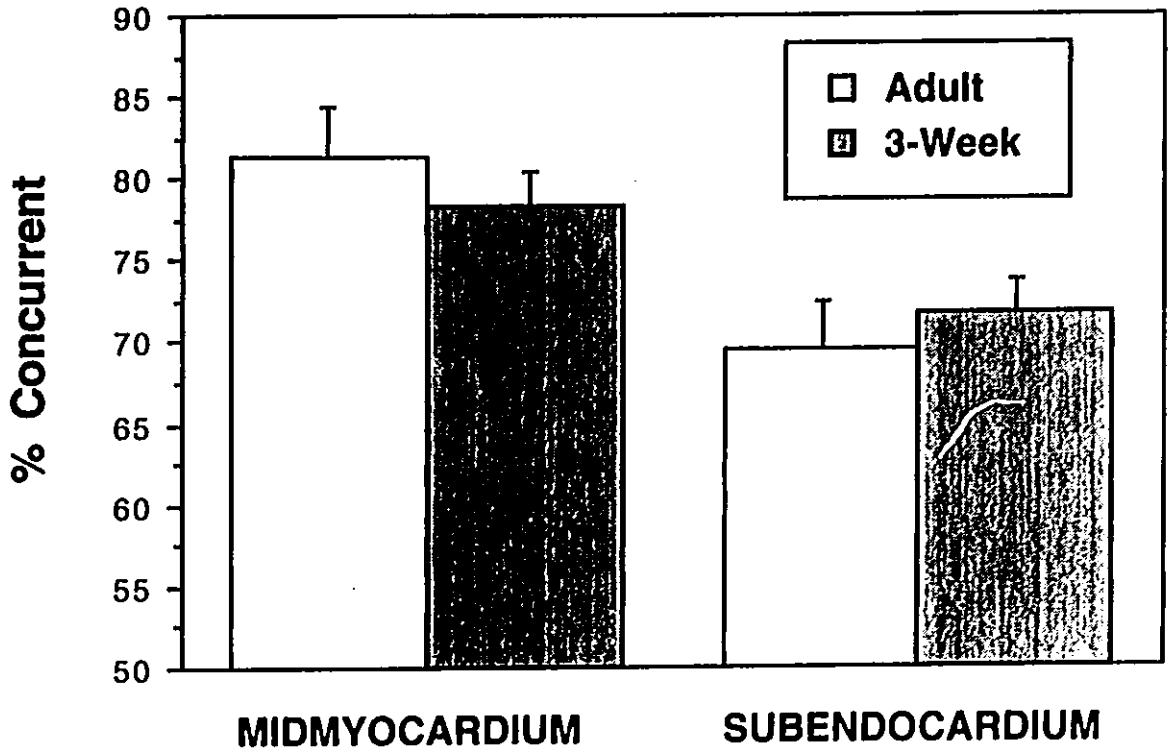


Figure 15. Graphical representation of the effect of regional differences on the % of concurrent flow within the ADULT and 3-WEEK old hearts. Results were significanly different ($p<0.05$). However, regional comparisons between groups were not statistically significant. Bars represent means ± SEM.

DISCUSSION

The preeminent finding of this investigation was that coronary capillary flow direction can also characterized with success in the isolated perfused heart model. This finding resulted from application of our previously described technique of sequential in vivo infusion of different colored microspheres into rat myocardium.

The Langendorff heart offers a comparative ease of preparation and certain technical advantages next to an *in vivo* model. The ability to precisely control experimental parameters and maintain stable mechanical function over extended periods is clearly superior to that possible under in vivo conditions (see Neely et al. 1975; de Leiris et al, 1984). For the purposes of the present study, the infusion of microspheres via the ascending aorta into the coronary vasculature was considerably less difficult compared to in vivo infusion into the left atrium. In fact, *in vivo* infusion of microspheres in the case of the smaller developing heart proved technically unfeasible. This approach also permits direct microsphere entry into the coronary vasculature with, moreover, only 5-6% of the number of microspheres needed for comparable numbers of flow vector observations in the intact heart. To validate the model, results obtained from our in vivo method presented in Chapter 3 were used as standards for comparison with those obtained in the present study.

Overall, flow vector data obtained from the isolated heart model do not appear appreciably different from results obtained in previous *in vivo* experimentation. As with the in vivo model, results indicate a predominance in concurrent flow direction, with a progressive decrease in the percentage of concurrent flow

with capillaries increasingly removed from an individual reference vessel. This was postulated in Chapter 3 to be representative of a distinctive geometric arrangement of arteriolar units; individual arteriolar units being characterized by a predominantly concurrent internal flow pattern, which, however, may be oriented in countercurrent fashion to adjacent arteriolar units. Therefore, as one expands the area of analysis away from a reference capillary, the greater the possibility of capturing capillaries originating from a separate arteriolar unit. In addition, the percentage of concurrent flow was significantly lower in subendocardium than in midmyocardium. Overall percentages of concurrent capillary flow direction over the range of capillary rankings in subendocardium were higher in the isolated heart than in vivo. This apparent discrepancy may be related to basic differences in mechanical function between the two models. Although regional capillary flow direction in myocardium is largely dictated by an intrinsic microvascular architecture, temporal variability in flow pattern may be produced by changes in contractile state, driving pressure, vasomotion, etc. In the Langendorff preparation, although the heart contracts (isovolumetrically), its mechanical performance and metabolic requirements do not approach those of the intact heart *in vivo*. In the intact heart, the balance between oxygen supply and demand is more critical in the subendocardial layers than in subepi- or midmyocardium (Griggs et al.,1973; Eng et al., 1987). Such regional differences would be expected to be minimized in the Langendorff model, where a depressed myocardial contractile state is less likely to influence microvascular flow pattern.

A further objective of this study was to compare regional capillary flow direction in young versus adult hearts. Our rationale behind this objective is based on findings that the capillary network in developing hearts depicts systematic differences in geometric organization relative to adult hearts. For example, a lateral expansion of the capillary set with myocardial growth is evidenced by an increase in the number of adjacent capillaries traversing the path from arteriole to venule (Batra and Rakusan, 1992). In terms of flow direction, this conceivably increased the possibility of encountering countercurrent flow observations in the younger heart if "individual" capillary sets were to display a uniform internal flow pattern. Despite slight qualitative differences, results were not significantly different from those obtained in adult myocardium. This would suggest that capillary flow direction does not differ appreciably between the two age groups. Thus, the typical lateral expansion of the coronary capillary set with age is not reflected by adaptations or changes in flow direction subsequent to the period of post-weaning development.

Unfortunately, we were not successful in obtaining reliable data in animals prior to 21 days of age. Whether any differences in capillary flow direction in comparison to older animals would prevail during this more proliferative phase of capillary growth cannot be answered at present.

In conclusion, what this study indicates is that the isolated heart model according to Langendorff is a suitable and useful tool for the determination of capillary flow vectors in rat myocardium. Despite slight differences noted in subendocardial regions between the two preparations, the general agreement with

our earlier *in vivo* results was encouraging. Indeed, the success of this approach prompted us to pursue the determination of capillary flow direction during conditions of experimental hypoxia using our colored microsphere technique. This study is described in the following chapter.

CHAPTER 5: THE EFFECT OF HYPOXIA ON CAPILLARY FLOW DIRECTION IN THE ISOLATED PERFUSED RAT HEART

INTRODUCTION

In Chapter 4 we indicated that the isolated perfused heart model was a valid and reliable method for the evaluation of coronary capillary flow direction. Isolated hearts from small mammals, particularly rats, have been used extensively to gain knowledge about the myocardial consequences of ischemia and hypoxia. Experimental hypoxia has been utilized by investigators to examine for example, cardiac mitochondrial energy states through examination of NADH fluorescence patterns (Steenbergen et al., 1977; Ince et al., 1993), or to study myogenic and flow-dependent autoregulatory mechanisms in the heart (Döring and Dehnert, 1987). It must, of course, be recognized that ischemia and hypoxia are not synonymous, and that the consequences of various types of hypoxia are not necessarily the same as the consequences of ischemia. In ischemia, the reduction of myocardial perfusion results not only in hypoxia, (i.e., an insufficient oxygen supply to meet the oxidative requirements of the tissue) but also in reduced supply of metabolic substrates and reduced removal of catabolites. On the other hand, during hypoxia with continued perfusion, lactate accumulation and cellular acidosis may be prevented, thereby allowing anaerobic glycolysis to continue as long as substrate remains available (Rovetto et al., 1973; Neely et al., 1975). Nevertheless, hypoxia is probably the most important component of ischemia. Myocardial hypoxia without reduced coronary blood flow can occur at high altitudes, in severe

anemia, or in certain types of poisoning. Mild but persistent degrees of hypoxia, which can be caused by a variety of conditions including cyanotic congenital heart disease or severe anemia, may also cause progressive cardiac damage and cardiac failure.

The effects of hypoxia on the coronary microvasculature have, to date, not been fully elucidated. Inhibition of the oxidative metabolism of the myocardium tends to result in a compensatory dilatation of coronary arteries and arterioles, with a corresponding increase in coronary flow. According to current opinion, it is likely that dilatory mediators are liberated from the vascular endothelium (for a review, see Jones et al., 1993). With respect to recruitment of capillaries in the coronary microcirculation, a degree of inconsistency and controversy has arisen. An increase in the density of perfused capillaries (functional recruitment) has been postulated by some authors as an important (eg Martini and Honig, 1969; Henquell et al., 1977; Rose et al., 1980), or more moderate (Rakusan and Korecky, 1982) compensatory process in myocardium under hypoxic conditions. However, the concept of a functionally significant capillary reserve has not been universally accepted (eg, Steinhausen et al., 1978; Schmid-Schönbein, 1990; Vetterlein et al., 1993).

The spatial distribution of capillary networks in myocardial tissue and the direction of blood flow therein (concurrent vs countercurrent capillary flow) has also been regarded as a potentially important determinant of tissue oxygenation (Diemer, 1965; Lübbers, 1976). Capillary flow direction has been characterized *in-vivo*, in normal adult rat myocardium utilizing the colored microsphere tech-

nique (Reeves and Rakusan, 1988) and as illustrated in Chapter 3, with respect to regional differences in normal hearts and hearts subjected to pressure overload hypertrophy . In Chapter 4 we further validated the determination of capillary flow direction in the isolated perfused Langendorff heart model, in both young and adult rat myocardium.

What was unknown, however, was whether acute experimental interventions such as severe hypoxia could result in alterations in coronary capillary flow pattern in the face of a significantly lowered arterial PO_2. To this end, we took advantage of the technical conveniences and superiority of the Langendorff preparation to directly evaluate the effects of acute oxygen deficiency on the coronary microcirculation, and utilized our colored microsphere technique to characterize regional capillary flow direction.

METHODS AND MATERIALS

Experimental animal preparation:

To investigate regional capillary flow direction in hypoxic rat myocardium an isolated perfused "Langendorff" model was utilized for the sequential infusion of distinctly colored microsphere suspensions. Experimental preparation of the heart essentially followed the methods outlined in the previous chapter. For the initial series of experiments (Series A) utilizing three differently colored microsphere aliquots, male Sprague-Dawley rats 350-400 g were randomly divided into two groups: those exposed to acute hypoxia (n=12), and their matched

controls (n=10). Additionally, a second (n=5) series of experiments (Series B) utilizing two separate colors of microspheres was subsequently carried out with animals subjected to hypoxia.

As before, hearts were perfused at a constant pressure (80 cm of H_2O) with Krebs-Henseleit bicarbonate buffer. For the present series of experiments, the perfusate was equilibrated with 95%O_2-5%CO_2 or 95%N_2-5%CO_2 (to produce experimental hypoxia) and warmed to 37°C in a water-jacketed column.

In all animals, following anesthesia with sodium pentobarbital (0.1ml/100g body weight), a midline laparotomy was performed with the inferior vena cava being isolated. Heparin (0.1ml/100 g body weight) was then injected into the vena cava and allowed to circulate for one minute. Subsequently, the abdominal aorta and inferior vena cava were severed to bleed the animal. Once breathing had ceased, the heart was exposed and loosely packed with crushed ice to arrest it. The aorta was then isolated with a small incision made below the branch of the right carotid artery. A stainless steel cannula primed with saline (containing 0.5 mM CaCl2) was inserted into the aorta with a 4/0 silk suture tightened around the aorta and cannula. The heart was quickly excised with the cannula and attached to a Langendorff perfusion apparatus. Hearts were perfused at a constant pressure (80 cm of H_2O) with Krebs-Henseleit bicarbonate buffer equilibrated with 95%O_2-5%CO_2 or 95%N_2-5%CO_2 and warmed to 37°C in a water-jacketed column.

A small, compliant balloon-tipped catheter (deflated) was placed in the left ventricle and held in place by the mitral valve. A small, known volume of water

was added to the balloon in order to produce an end-diastolic pressure of approximately 6 mmHg. The peripheral end of the catheter was attached to a transducer (Statham Db 23) and recorder (Grass Model 7) to monitor left ventricular developed pressure, and dP/dt continuously. The hearts were electrically stimulated at a constant rate of 300 beats/minute using small, spiral electrodes connected to the base of the right ventricle. Coronary flow rate (effluent from the right ventricle) was measured using a graduated cylinder.

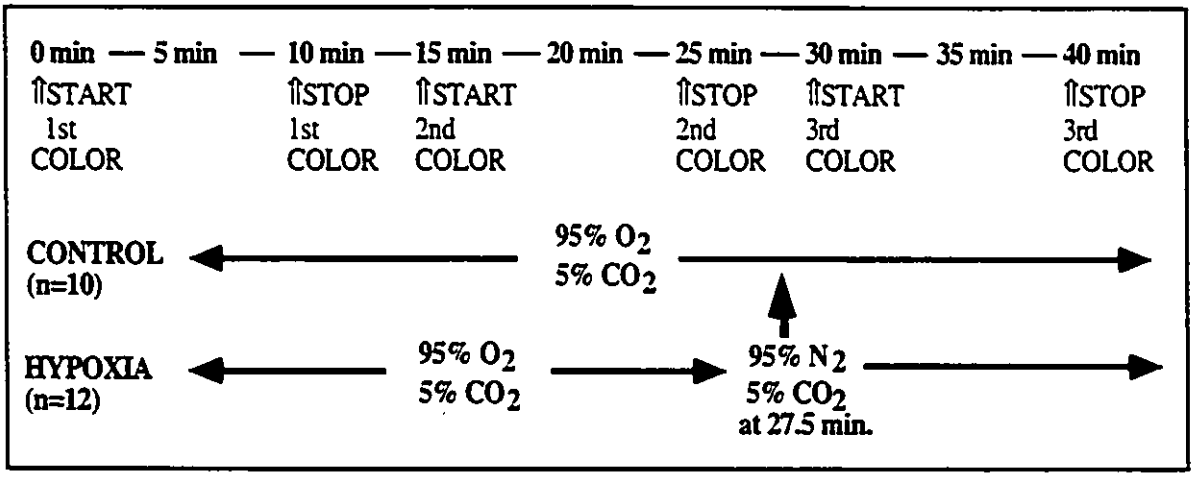
Colored microsphere procedure:

Three different colors of non-radioactive microspheres (red, blue, and green) with diameters of 10 ± 0.23 μm were utilized for coronary infusion. Prior to injection, the microspheres were suspended at a concentration of approximately 6 x 10^5/ml in saline containing 0.01% Tween solution, and lightly agitated in a vortex mixer for one minute. This was followed by brief (30-sec) sonication with a low-power ultrasound probe. Microsphere aggregates were not visible upon microscopic examination of the suspensions.

Subsequently, differently colored 2ml microsphere aliquots, each containing 6 x10^5 red, blue or green microspheres, were infused sequentially into the ascending aorta (retrograde perfusion to enter the coronary vasculature) at constant and identical rates of 0.2 ml/min for 10 minutes via a Sage Instruments Model 355 syringe pump. A 5-minute interval elapsed between injections. For the initial

series of experiments (Series A), a "control" group received three infusions of differently colored microspheres in sequence under normoxic perfusion conditions (95% O_2-5% CO_2). In the "hypoxic"group, the initial two microsphere infusions were conducted under identical perfusion conditions. Subsequently, at the midpoint between the 2nd and 3rd set of microsphere infusions (i.e., at approximately 27.5 minutes into the experiment), a state of experimental hypoxia was created. The perfusate was gassed with 95% N_2-5% CO_2 in exchange for 95% O_2-5% CO_2. Hearts continued to be perfused in this manner until the infusion of the final aliquot of microspheres was concluded.

(see scheme below)



A second series of experiments (Series B) served as a "quality control" study to substantiate the actual effects of hypoxic perfusion. This experimental modification consisted of two 10-min microsphere infusions. The initial infusion was

conducted under normally oxygenated perfusion conditions. The second infusion was performed under conditions of experimental hypoxia.

After the microsphere infusions were completed, the hearts were weighed, dissected, and frozen in liquid nitrogen for cryotomic preparation of 40 μm longitudinal sections from midmyocardial and subendocardial regions of the left ventricular free wall.

The rationale behind the use of the colored microsphere technique for the characterization of capillary flow direction has previously been described in Chapter 3. The sequential infusion of three differently colored microsphere suspensions results in the formation of four distinct aggregation patterns within the coronary microcirculation: (1) Single sphere of any microsphere color (2) Aggregates of one microsphere color only (3) Aggregates containing two microsphere colors (4) Aggregates containing three microsphere colors. According to aggregation patterns, a "flow vector" is established based on the sequence of microsphere colors entrapped within each capillary (Figure 16). Detailed microscopic examination of flow vectors obtained from neighboring capillaries enables the characterization of regional capillary flow direction.

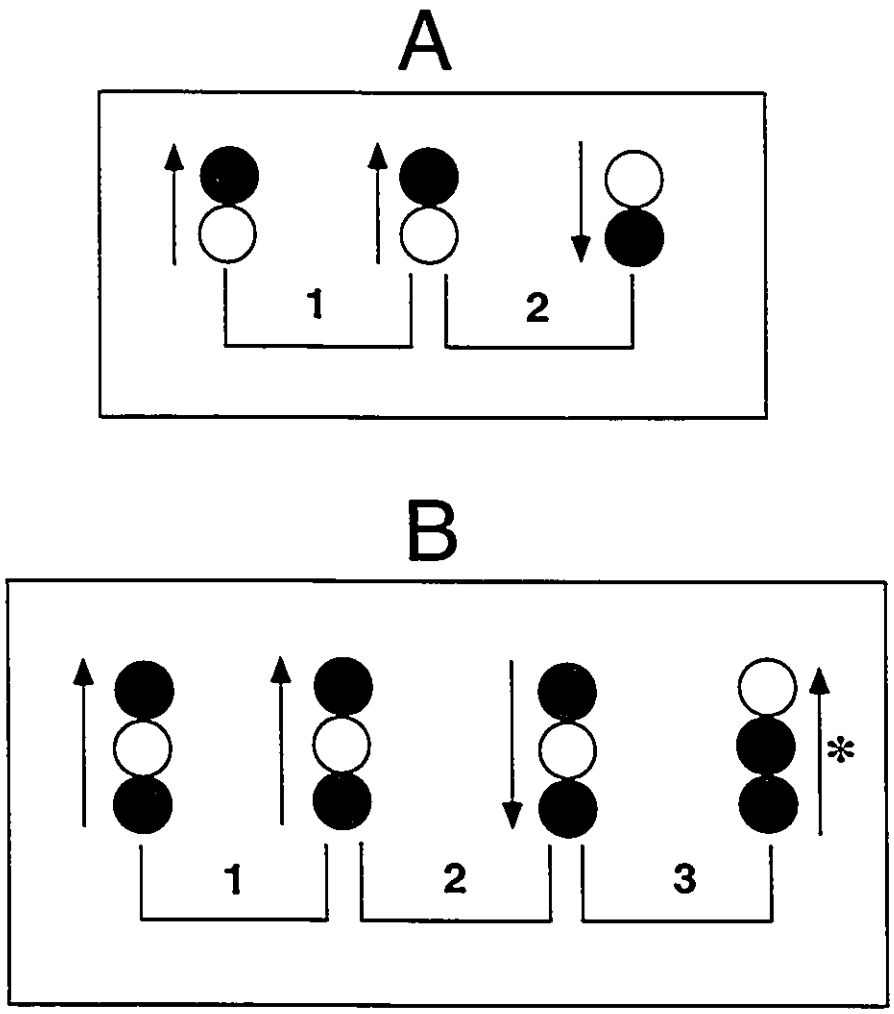


Figure 16. Differently colored microspheres red, blue, and green (illustrated by black, white and grey circles respectively) showing "flow vectors" based on the sequence of colors infused; Black first >White second > Grey third. Arrows indicate flow direction. **PANEL A:** based on serial infusion of two microsphere colors (1) concurrent flow between capillaries, or (2) countercurrent flow. **PANEL B:** based on serial infusion of all three microsphere colors, (1) concurrent flow between neighboring capillaries or (2) countercurrent flow. Both observations 1 & 2 based on sequence of infusion; black>white and white>grey. (3) countercurrent flow, due to reversal of sequence in one capillary* from concurrent black>white to countercurrent white>grey.

Statistical analysis:

To investigate possible differences within and between the two groups in experimental Series A, a 3-way nested factorial ANOVA for proportions, utilizing an arc sin transformation of the dependent variable data (Armitage and Berry, 1987) was the procedure of choice to investigate the contributions and potential significance for the independent variables of control versus hypoxia (CON/HYP), midmyocardium versus subendocardium (MID/SUB-E), and the sequence of microsphere colors within capillaries to the flow vector observations. The individual hearts were regarded as a "nested" factor within the independent variable of control/hypoxia.

A Student's *t*-test was employed to compare the results of the 2-color flow vector observations from experimental Series B, with the data from the 2-color normoxic perfusion interval of the "hypoxic" group in experimental Series A.

RESULTS

The changes in coronary flow with respect to time, during the serial infusion of three differently colored microsphere aliquots (Series A), are illustrated in Figure 17. This figure further serves as a flow chart for the microsphere infusion protocols used for both the control and hypoxic groups. Coronary flow rate (ml/min/g) was not significantly different between groups during the first two microsphere infusions conducted under normal perfusion conditions. Upon induc-

tion of experimental hypoxia (95%N_2-5%CO_2) in place of (95%O_2-5%CO_2), a significant increase in coronary flow occurred, compared to the same time interval in controls ($p<0.01$), at the 30-min mark. By the 35-min mark, coronary flow had substantially decreased, but was still significantly greater than in controls ($p<0.05$). However, by the 40-min point coronary flow had fallen significantly below control values ($p<0.01$).

Similar measurements were performed in the series of 2-color microsphere experiments (Series B). In these experiments (see Figure 18) coronary flow remained stable during the 10-min microsphere infusion period under normally oxygenated conditions. Hypoxic perfusion (95%N_2-5%CO_2) was subsequently induced at the midpoint between the two infusion periods. By the 15-min mark, coronary flow had increased significantly above levels seen during normoxic conditions ($p<0.001$). By the 20-min mark coronary flow had dropped below the 15-min peak value ($p<0.05$), and a further slight, though non-significant decrease was seen by the end of the experiments. Notably, coronary flow throughout the period of microsphere infusion under hypoxic conditions was consistently, and significantly higher than during the period of hypoxia for experiments utilizing three microsphere colors.

Although distinct microsphere aggregate patterns as described above can occur within individual coronary capillaries, the determination of capillary flow vectors among neighboring capillaries required observing aggregates of at least two different colors. Figure 19 illustrates the possible combinations of concurrent

capillary flow patterns determined by the sequence of colored microsphere aggregates in controls and in the experimental hypoxia group for the 3-color microsphere experiments.

Overall results for both groups indicated a predominance in concurrent flow direction among neighboring capillaries. However, analysis of variance further revealed that total percentages of concurrent flow were lower ($p<0.01$) in the "hypoxic" group than in controls (Figure 19).

As described above, the serial injection of three separate microsphere aliquots of different colors creates specific aggregation patterns within coronary capillaries (see Plate 5, and Figures 16, 19). Percentages of concurrent flow based on the possible sequences of microsphere colors revealed significantly lower values in hypoxic hearts than in controls ($p<0.01$). However, since the initial two colors of microspheres were injected under normal perfusion conditions in both groups (Figure 19), it was necessary to separate further the 2-color aggregates into A) initial sequence only, (i.e., the first two sequentially infused microsphere colors), and B) mixed (i.e., containing the last injected color and either the first or second of the earlier injected colors). This comparison revealed no significant differences in the control group. However, the "hypoxic" group revealed a significant decrease in percentage of concurrent flow between the 2-color aggregates formed under normoxic conditions, and the 2-color aggregates formed under conditions of experimental hypoxia ($p<0.01$) (Figure 19). No statistically significant differences were revealed among any of the microsphere aggregate combinations in the control group. The decreased percentages of concurrent flow

during hypoxia were reflected histologically by a change in orientation of the later infused microspheres relative to the positions of the previously infused colors (see Figure 16b).

Figure 20 illustrates the effect of regional differences i.e., midmyocardium vs subendocardium (MID/SUB-E) on percentages of concurrent flow in control and hypoxic hearts. For both groups, subendocardial regions were characterized by significantly lower percentages of concurrent flow than in midmyocardium ($p<0.01$).

In experimental Series B, two microsphere infusions were conducted, the initial under normally oxygenated conditions and the second during hypoxic perfusion. As the initial two microsphere colors in Series A (as described above) were infused under normal O_2 perfusion conditions, the flow vectors formed during this period were further used for comparison with the present 2-color hypoxia observations (Figure 21). These results indicated a lower overall percentage of concurrent flow in the 2-color hypoxic group (Series B) when compared with the earlier 2-color normoxic perfusion period in SERIES A (Student's *t*-test; $p<0.05$).

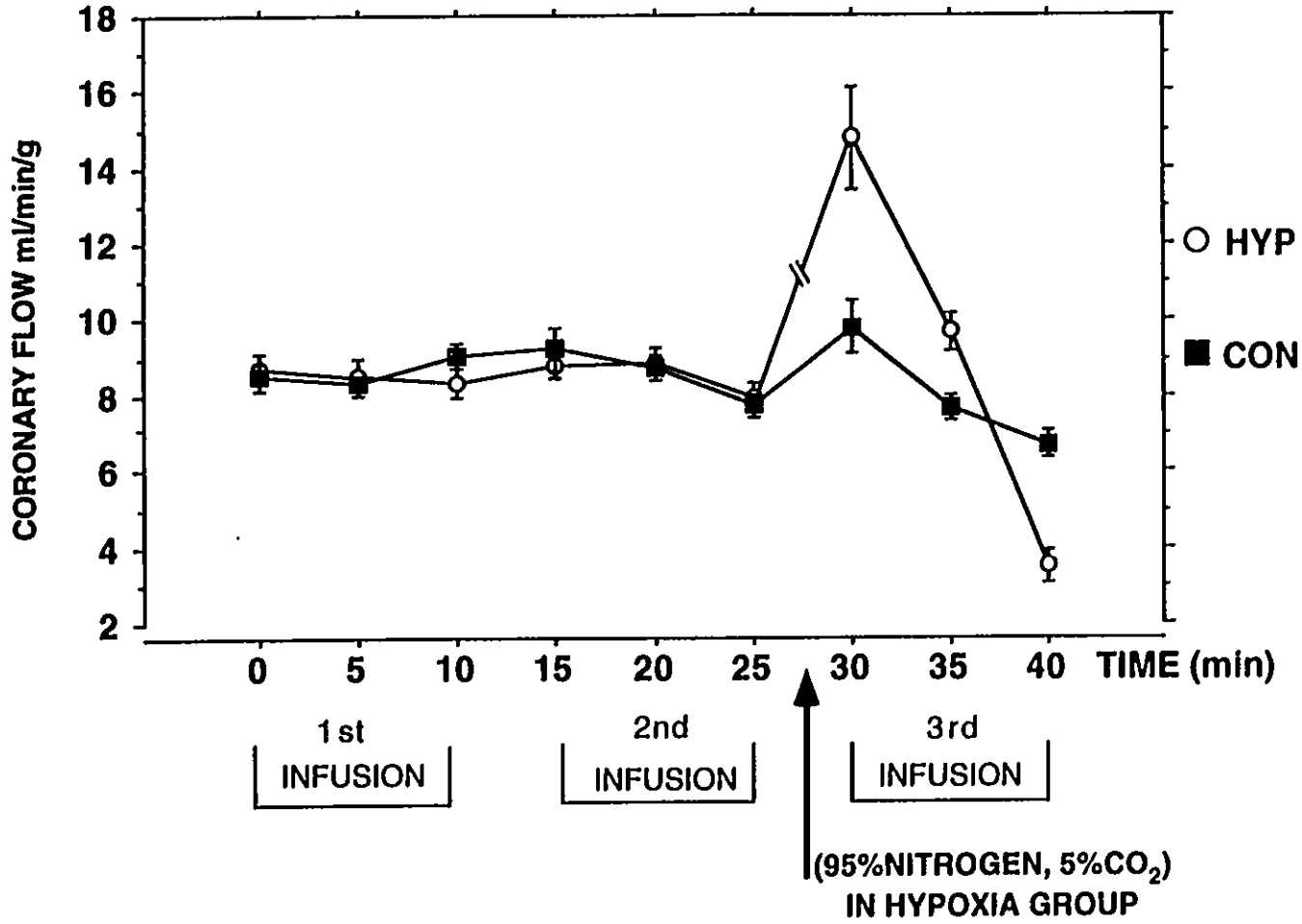


Figure 17. Graphical record of coronary flow rate (ml/min/g) in control (CON, n=10) and experimental hypoxic (HYP, n=12) groups in **Series A**. Note stability of coronary flow during infusion of initial two microsphere aliquots in both groups; perfusate saturated with (95%O_2-5%CO_2) in both groups. Upon induction of hypoxia (95%N_2-5%CO_2) in the HYP group, flow increased significantly above control values ($p<0.01$). By endpoint of final microsphere infusion, flow had fallen significantly below control values ($p<0.01$). Points represent means ± SEM.

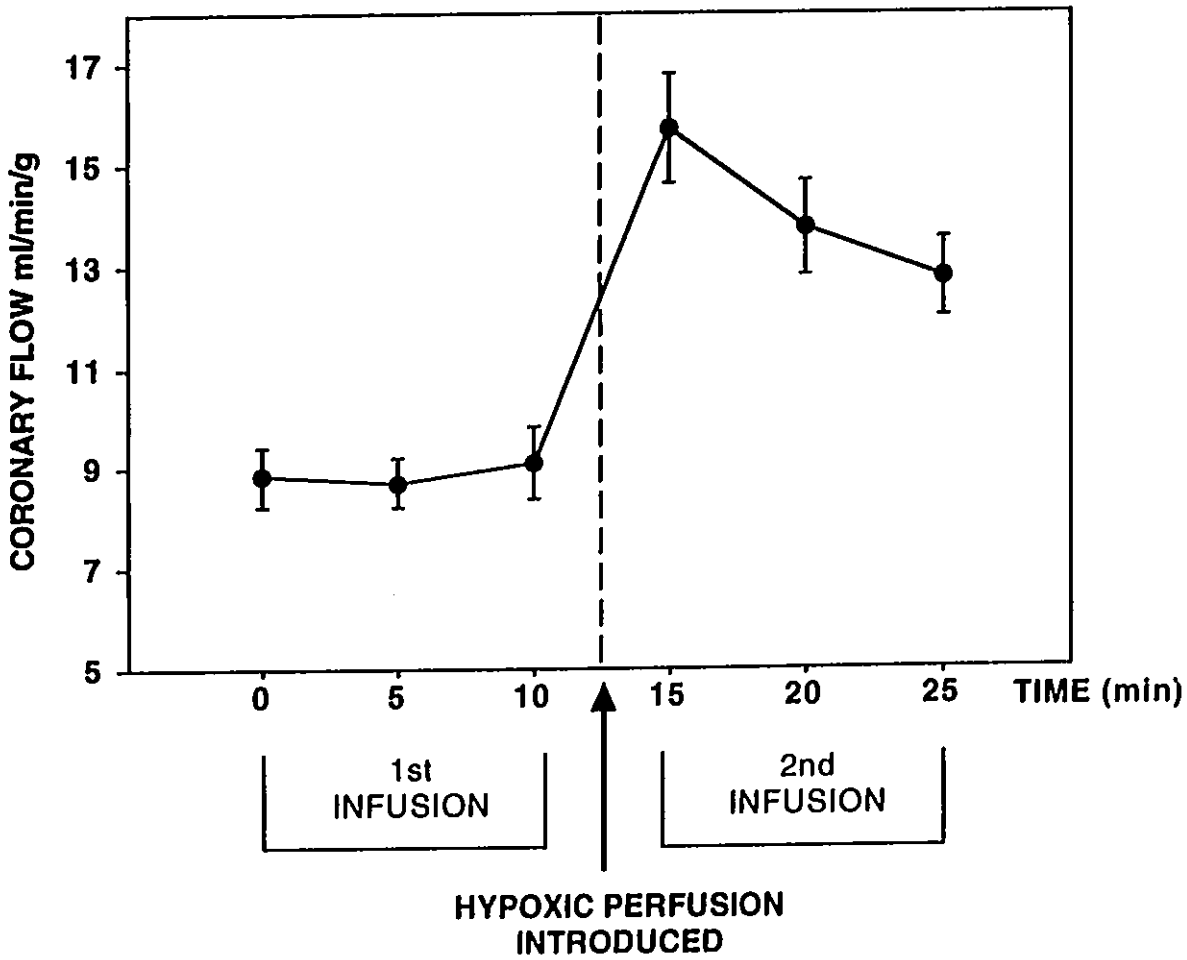


Figure 18. Graphical record of coronary flow rate (ml/min/g) in two color "hypoxic" group, Series B. Initial microsphere aliquot infused under normoxic conditions ($95\%O_2$-$5\%CO_2$), with the second during hypoxic perfusion ($95\%N_2$-$5\%CO_2$). Points represent means ± SEM.

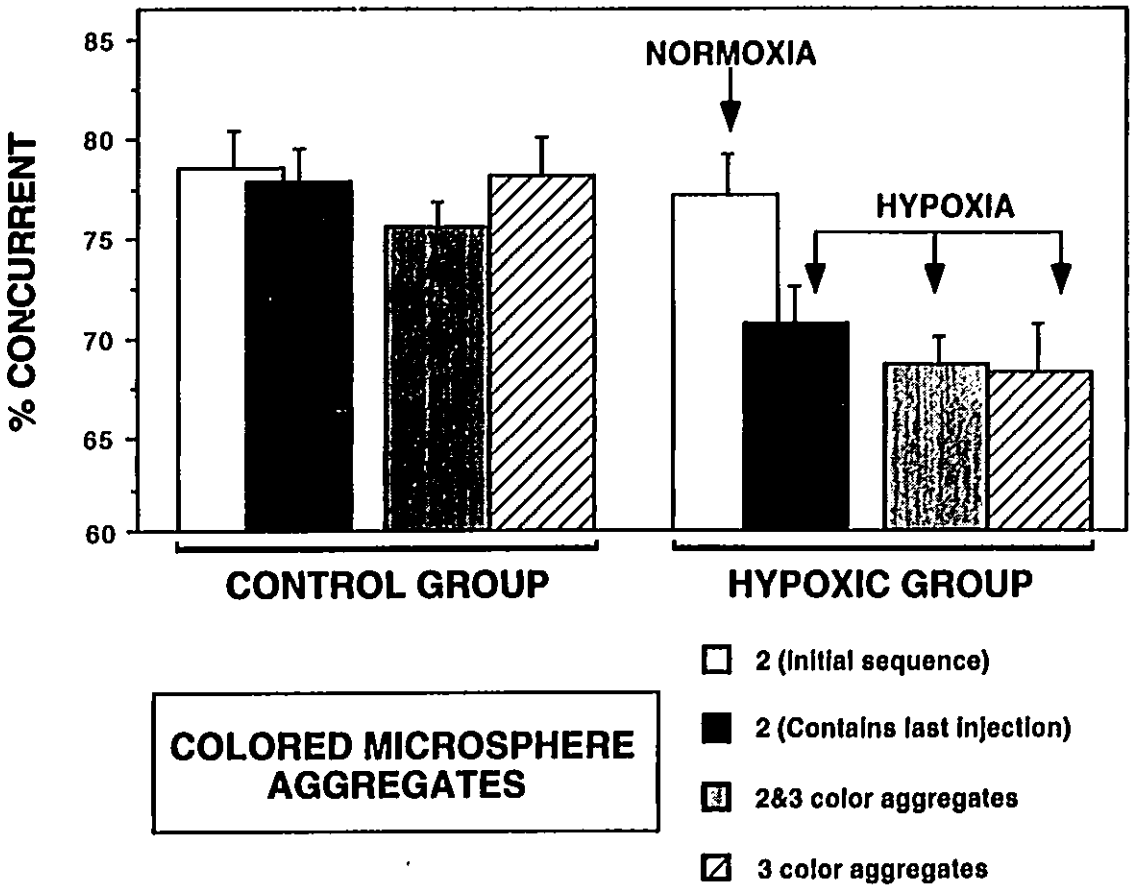


Figure 19. Concurrent flow based on aggregates derived from 3 colors of microspheres (red, blue, and green), Series A. **White bars:** 2-color aggregates containing initial 2 (red,blue) microspheres. **Black bars:** 2-color aggregates containing final color (green), with either first or second colors of microsphere. **Grey bars:** 2 and 3- color aggregates, one capillary containing all colors, the other containing any 2 colors. **Hatched bars:** aggregates containing all 3 colors of microspheres. **CONTROL GROUP:** no significant differences revealed. **HYPOXIC GROUP:** lower proportions of concurrent flow revealed among all aggregates formed during hypoxia than during normoxic perfusion ($p<0.01$). Bars represent means ± SEM.

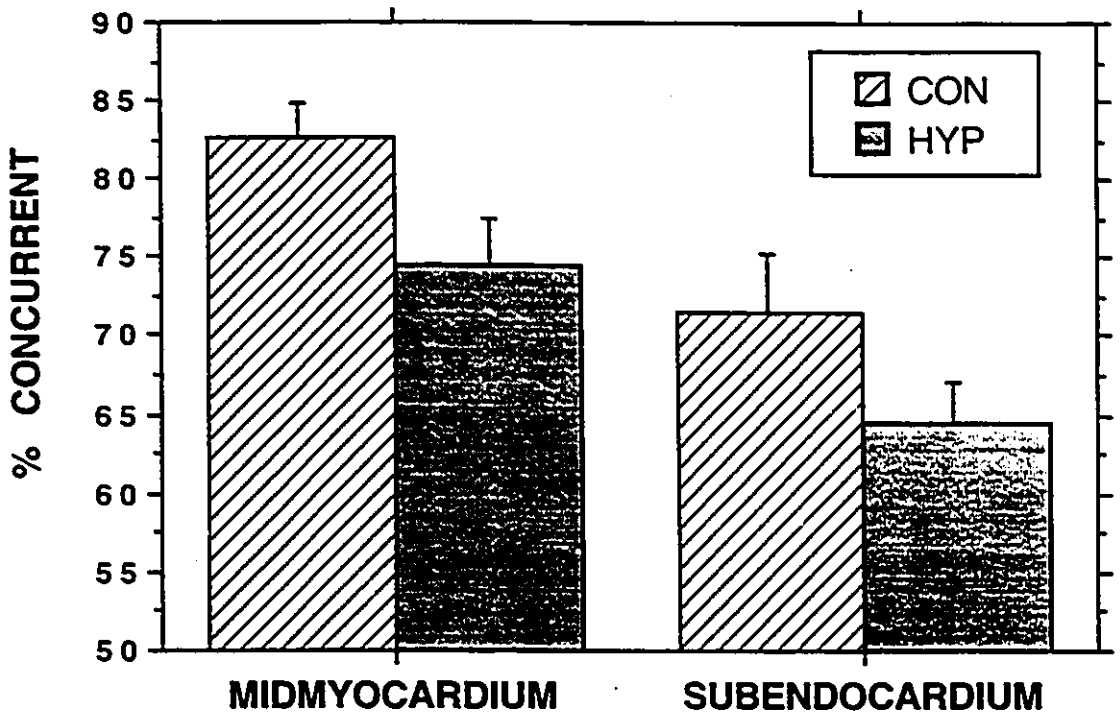


Figure 20. The effect of regional differences: midmyocardium vs subendocardium on percentage of concurrent flow for control (CON) and hypoxic (HYP) groups, Series A. In both groups, the percentages of concurrent flow were significantly greater in MID regions than in SUB-E regions ($p<0.01$). Bars represent means ± SEM.

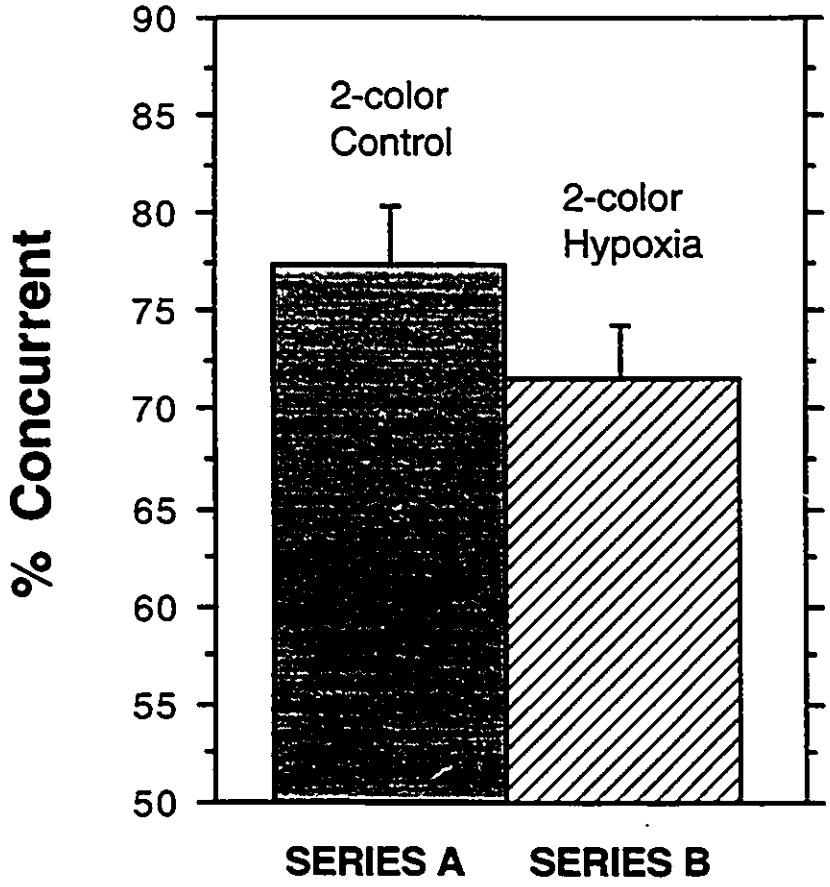


Figure 21. Graphical comparison of the 2-color (red and blue microspheres) hypoxia data with 2-color normoxic perfusion data. These results indicated a lower overall percentage of concurrent flow in the two color hypoxic group (Series B) when compared with the earlier two color normoxic perfusion period in SERIES A (Student's t-test; $p<0.05$). Bars represent means ± SEM.

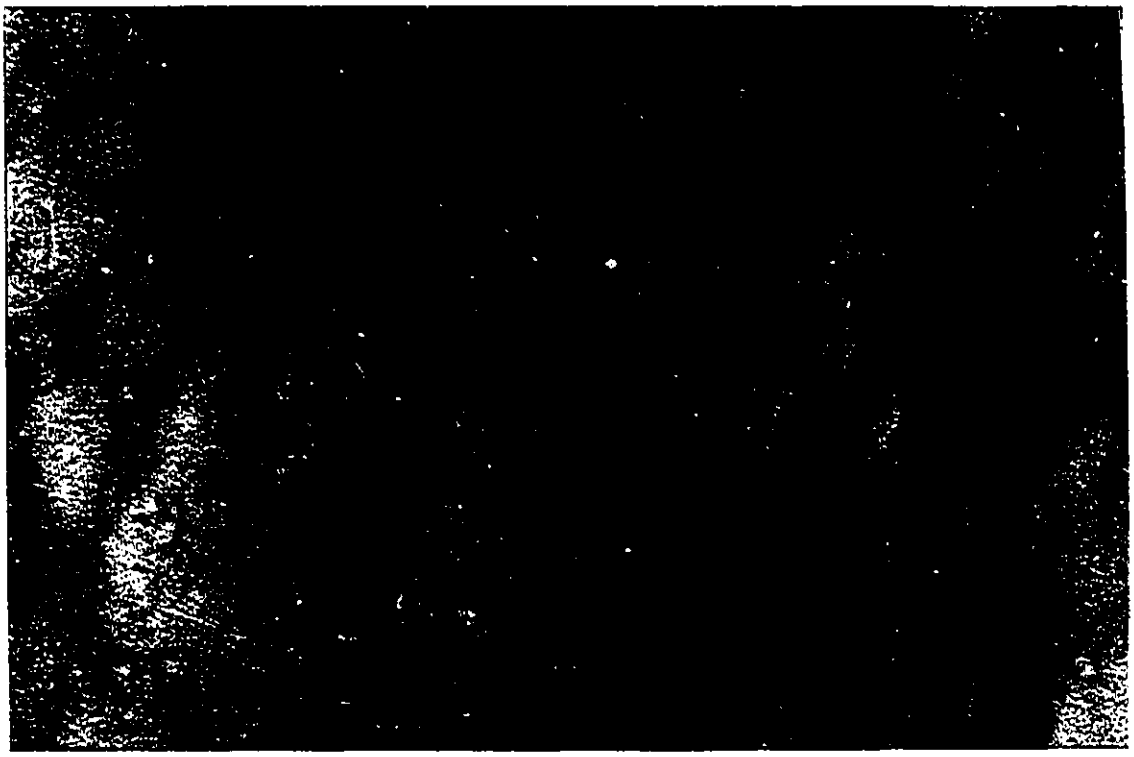


Plate 5 Photomicrograph illustrating capillary "flow vectors" in adjacent capillaries based on the sequential infusion of three separate colors of microspheres in the isolated perfused rat heart. Mag. 500x

DISCUSSION

Compared with an *in vivo* model, the ability to control more rigorously experimental parameters and conditions in a highly reproducible fashion prompted us to pursue the current study in the isolated heart for determining capillary flow direction during conditions of experimental hypoxia using our colored microsphere technique. The isolated heart model provides a more precise and effective means of inducing an acute hypoxic state by using nitrogen instead of oxygen in the gas mixture with which the perfusate is saturated.

The principal finding of the present investigation is that acute interventions, such as those produced through induction of experimental hypoxia, may modify the normal flow pattern existing among neighboring coronary capillaries. As previously described, the sequential infusion of three separate microsphere aliquots of different colors creates specific aggregation patterns within the coronary microcirculation. Based on the sequences of microsphere colors entrapped there was a higher percentage of countercurrent flow revealed among neighboring capillaries subsequent to the hypoxic intervention than during normoxic perfusion ($p<0.01$). These findings were reflected by a change in position of the later injected microspheres relative to the positions of the previously injected microspheres. Specifically, microspheres infused during the period of hypoxia were often found ahead of those microspheres infused under normal perfusion conditions (Figure 16b).

To investigate the possibility that any change in orientation of microspheres comprising the latter injection may simply be due to the cumulative effect of three

sequential infusions per se, a three color group serving as control received all infusions under normal perfusion conditions. Within this control group, there were no significant differences in percentages of concurrent flow based on 2-color or 3-color observations. Notably, results indicated that the percentages of concurrent flow based on three microsphere colors were significantly different between the control and "hypoxic" groups. This suggested that the period of hypoxic perfusion had modified the flow pattern with respect to the final injected microspheres. However, this period of infusion was accompanied by two distinctly contrasting time courses. Initially, coronary flow increased significantly at the beginning of the third series of microsphere infusions. It had decreased in an equally significant fashion by the termination of the final infusion. From these data on coronary flow, it becomes extremely difficult to establish the potential contributions of these distinct flow periods to the establishment of the resulting capillary flow pattern. In other words, the change in orientation of the final injected microspheres could have occurred during the period of higher flow, with the possible mobilization of either previously nonperfused capillaries (eg Martini and Honig, 1969; Rose et al., 1980) which now contain microspheres, or through an amplification of capillary flow velocities during hypoxia (Vetterlein et al., 1993). Alternatively, one cannot rule out the possibility that this occurred during the latter period of low coronary flow which was accompanied by rapidly diminishing cardiac function. We also observed histologically a greater number of larger microsphere aggregates comprising the final infusion. This points to the possibility of localized ischemia

through occlusion of larger arterioles as a contributing factor to an altered capillary flow pattern.

In other experiments (not reported) the duration of hypoxia was increased by an additional five minutes before infusion of the final set of microspheres to examine the time course of diminishing coronary flow, as well as microsphere aggregation patterns. These experiments disclosed that three sequential infusions of microspheres were incompatible with hypoxic perfusion of a more extended duration. With six hearts, ventricular failure occurred during infusion of the final set of microspheres. Certain regions of tissue revealed noticeable microsphere aggregation, probably creating regions of severe localized ischemia.

The results from the series of 3-color microsphere experiments as described above were not successful in elucidating whether the period of high flow hypoxia or the period of diminished flow, "ischemic" hypoxia was responsible for the apparent flow reversals evidenced histologically by microsphere reorientation. These findings prompted us to perform an additional series of experiments consisting of two microsphere infusions only, the initial infusion under normal perfusion conditions, and the second under hypoxic conditions (Figure 18). As with the earlier 3-color experiments, coronary flow initially experienced a steep and predictable increase. However, in contrast to the earlier experiments, coronary flow remained elevated over that during the period of normoxic perfusion, and was significantly higher than during the period of hypoxia for experiments utilizing three microsphere colors. Since the initial two sets of microspheres in the 3-color hypoxia experiments were infused under normoxic

perfusion conditions, it was deemed feasible to compare the flow vector observations made during this time period with the results from the present 2-color hypoxia group. These results revealed a significantly lower percentage of concurrent flow in the 2-color hypoxia group ($p<0.05$). Furthermore, the overall proportions of concurrent flow were similar to those seen in the 3-color hypoxia group when all three microsphere color observations were considered. Thus, one may conclude that the decreased percentage of concurrent flow observed in both Series A and Series B was due primarily to the effect of hypoxia itself, rather than to ischemia.

An additional finding from the present study was that subendocardial regions in both the 3-color control and hypoxia groups showed significantly lower percentages of concurrent flow in the subendocardium than in midmyocardium ($p<0.01$). These results are in agreement with our previous investigations of coronary capillary flow direction. This has been postulated to be representative of a distinctive geometric regional organization of coronary microvascular units (see Chapter 3).

In summary, the results from the present study appear to be consistent with a model in which the direction of capillary flow may be affected by the local oxygen tension or gradients in the tissue. The actual mechanisms responsible for alterations in capillary flow direction during hypoxia (as revealed through the reorientation of microspheres within the microcirculation) remain at present unclear. Nevertheless, as pointed out by Lübbers (1976) and by Steinhausen and co-workers (1978), a mixed countercurrent-concurrent flow arrangement could

provide a more homogeneous pattern of oxygenation, which would be beneficial to tissue function under conditions of critical oxygen supply. The present findings could therefore be interpreted with caution as an immediate response or attempt at the level of the coronary microcirculation to sustain adequate myocyte oxygenation in the face of a severe reduction in the arterial PO_2.

CHAPTER 6: OVERALL SUMMARY AND CONCLUSIONS

The advent of the colored microsphere technique has provided us with a novel and practical approach to help clarify certain unresolved issues in the field of coronary microcirculation research.

In the introduction to Part A of this dissertation it was pointed out that the functional significance of myocardial borderzones has been a controversial topic for numerous years. In fact, intense debate over its very existence has resulted in a profusion of research studies utilizing a variety of techniques, none of which, to date, has provided any conclusive answers. The presence of a significant collateral blood supply and borderzone which could escape necrosis during coronary infarct progression could, at least theoretically, enhance the possibility of tissue salvage through the use of appropriate medical intervention directed at increasing collateral blood flow and/or reducing oxygen consumption. However, previous studies have demonstrated ambiguous and contradictory data based upon biochemical gradients and blood flow as a function of distance from a central ischemic core. Other, perhaps more innovative attempts to resolve this controversy on the basis that the presence of a functionally significant borderzone required a demonstrable microcirculatory system capable of infarct response, have also produced conflicting results. Notwithstanding, all of these previous studies appear to be at least consistent with a more complex model of microcirculatory architecture and function than previously considered. Clearly, the concept that the area at risk contained a functionally viable borderzone needed experimental support.

We proposed that this was plausible if indeed microvascular anastomoses cross the interface and connect the beds of adjacent source arteries, so that potentially jeopardized tissue across the interface had access to a dual arterial blood supply. Our non-radioactive colored microsphere technique provided us with the tools necessary to investigate this possibility (1) through microscopic analysis of perfusion field territories subsequent to simultaneous *in vivo* infusion of two different colored microsphere suspensions into adjacent coronary arteries, and (2) through the use of additional colors of microspheres infused after preliminary labeling, in order to investigate the temporal evolution of a perfusion field in response to an experimental intervention.

Our initial objective was to confirm the existence of microvascular anastomoses emanating from independent arterial sources. By utilizing the colored microsphere technique, we were able to identify unequivocally a specific interface region of canine myocardium that was perfused by both arterial branches. Since there were to our knowledge, no previous quantitative descriptions of coronary perfusion territory overlap, we defined and measured parameters considered important for a realistic characterization of microvascular anastomotic supply, ie., the ITZ and BWZ. Our experiments revealed an interface transition zone (ITZ) up to 5000 μm wide at the LAD-LCx interface where capillaries containing microspheres of one or the other color were intermingled. Within this region, a boundary watershed zone (BWZ) up to 3000 μm wide was found with capillaries containing microspheres of both colors. Vessels beyond the transition region contained microspheres of only one color, so flow through upstream collateral

vessels was probably not responsible for dual labeling of capillaries in the watershed zones.

Since it was improbable that the outermost vessels with a dual arterial supply would be consistently labeled, the width of the actual BWZ as determined in our study is likely an underestimate of the width of tissue having access to a dual source of perfusion. It is therefore plausible that "actual" zone dimensions are in fact intermediate between the measured ITZ and BWZ.

While these results strongly supported the notion that interarterial microvascular pathways may contribute significantly to total collateral blood flow, only empirical evidence from experiments designed specifically to address the question of how the interface responds to stresses such as coronary obstruction could answer this definitively. Such experiments were described in Chapter 2, Part A of this dissertation. For these experiments, watershed zones were initially labeled under equal pressure conditions, and then after the LAD artery was ligated, a third set of microspheres was infused into the patent LCx artery. Microspheres of the latter infusion were found to be distributed in significant numbers across the ITZ and BWZ. This confirmed our working hypothesis that the watershed zone would appear as part of the normally perfused region following obstruction of one of its parent arteries. Thus, 1) the boundary watershed zone may be protected from ischemia resulting from obstruction of either of its supply arteries, and 2) the boundary watershed zone is likely to be excluded from tissue considered "at risk" due to coronary artery obstruction.

We had furthermore hypothesized that the boundary watershed zone serves as a source of flow into the ischemic region. This may occur as a shift in the perfusion interface, indicated by an expansion of the normally perfused region, and by flow through microvascular collateral channels into the acutely ischemic region. To confirm our second hypothesis, we determined the specific locations at which the density of the third color of microspheres decreased significantly as a function of distance from the perfusion interface. Analysis of the distribution and density of the latter color of microspheres revealed a lateral extension of the perfusion interface into the occluded LAD region. The locations at which the densities of the latter spheres decreased abruptly were compared to the ITZ and BWZ boundaries, respectively. Detailed measurements revealed significant extensions of 24% and 48% respectively, into the LAD perfusion territory of these boundaries. These results confirmed our hypothesis that collateral flow included a significant microvascular component, and strongly suggested that the perfusion interface was both labile and amenable to manipulation during conditions of coronary occlusion.

Finally, all boundaries were significantly greater in subepicardial than in subendocardial regions. This additional finding provided a clue to transmural, and not merely radial dimensions along the coronary perfusion interface. This narrowing of the perfusion territory interface appears to be roughly proportional to the transmural geometrical configuration of the left ventricular wall, as the circumferential dimensions decrease approaching the endocardium. Nevertheless, it is reasonable to suggest that due to the particular vulnerability of the

subendocardium to a reduction in blood flow and hence ischemic injury, a more pronounced narrowing in this region might occur than in the outer myocardial layers, particularly under conditions which precipitate a coronary steal.

A natural extension of these experiments would be to investigate the effects of various coronary vasodilators on sources of blood flow to the boundary watershed zone, and the localization of the perfusion interface under such conditions. On the basis of our present results it would be reasonable to propose that the perfusion interface might shift when the parent artery supplying one perfusion territory is selectively dilated. It is likely that vasodilation of one bed will increase its microvascular pressures and, thus, make that territory dominant in supplying the boundary watershed zone. Regional coronary dilation could be accomplished by infusing a coronary vasodilator, either adenosine or nitroglycerine into one of the coronary arteries while perfusion pressure of both arteries is held constant. When coronary flow has increased 2-3 fold, a third color of microsphere would be infused into the dilated coronary bed and a fourth color of microsphere infused simultaneously into the other coronary artery. Similar experiments could also be conducted in hearts with acute coronary occlusion, with the addition of a different microsphere color infused into the patent artery during treatment with a coronary vasodilator. It would not be unreasonable to hypothesize that the dilator would increase microvascular driving pressure and therefore further extend the perfusion territory of the patent artery into the ischemic risk area. Since adenosine preferentially dilates the smaller coronary resistance vessels (with a distinct potential for initiating a coronary steal), and

nitroglycerine preferentially dilates the larger coronary conductance vessels (Cohen,1985) , their effects on the perfusion interface may differ. The effects of both of these prototype vasodilator agents on the localization and transmural dimensions of the coronary watershed zones would be examined in this protocol.

Experiments such as these would no doubt provide us with a better understanding of the supply of arterial blood to coronary microvascular elements. If coronary watershed zones could be demonstrated to protect ventricular function and metabolism from small artery obstruction and microembolism, then it may be possible to further enhance this salutary role through appropriate pharmacologic interventions.

Another practical application of our colored microsphere technique has been for the quantitative characterization of coronary capillary flow direction. Such information, until now, was not available for the myocardium. Although one may speculate about the nature of flow pattern on the basis of microvascular architecture, and while it is true that coronary capillaries can be visualized (in the subepicardium) through intravital microscopic techniques, these approaches are extremely limited and are not capable of providing realistic quantitative data pertaining to this potentially significant parameter for myocardial oxygen supply.

To this end, Reeves and Rakusan (1988) first utilized the capability of non-radioactive colored microspheres to depict capillary "flow vectors". This technique, by providing a permanent record of microvascular flow activity during the period of microsphere infusion allows for quantitative information to be obtained concerning myocardial capillary flow direction in a variety of exper-

imental environments. Our initial objective in these studies as described in Part B of this dissertation, was to quantitfy capillary flow direction *in vivo*, in both midmyocardial as well as subendocardial regions of rat myocardium. As the local contractile state and tissue pressures generated significantly affect subendocardial blood flow in comparison to epi- and midmyocardium flow, it was crucial to determine if significant regional differences existed in capillary flow direction. Furthermore, since the precarious balance between oxygen supply and oxygen demand could be exacerbated in hearts hypertrophied by a pressure overload, we also wanted to determine whether the overloaded myocardium would exhibit alterations in regional capillary flow pattern in an effort to sustain normal tissue oxygenation.

Histological analysis of normal and hypertrophic myocardial tissue revealed that numerous coronary capillaries contained microsphere aggregates based on the sequential *in vivo* infusion of two distinctly colored microsphere suspensions into the left atrium. We described a capillary "flow vector" on the basis of the sequence of colors trapped within each aggregate. Our results indicated a predominance in concurrent flow among neighboring capillaries, which decreased significantly over a range of capillary rankings. We hypothesized that this reflected the presence of discrete microvascular units, each exhibiting a predominantly concurrent internal flow pattern, which may occasionally be countercurrent to an adjacent microvascular unit. Thus, there was a higher probability in capturing countercurrent-directed capillaries available for histological sampling as the area of analysis from a reference capillary was expanded.

Accounting for the observed regional differences necessitated a greater degree of speculation, largely due to a limited knowledge regarding the architecture and distribution of intramyocardial arteriolar networks. It has been suggested (but not confirmed) that arteriolar vascular density is greater in the subendocardium. On the other hand, current evidence suggests that there is little difference in regional capillary density in the normal heart. If this is so, then it would follow that the arteriolar to capillary ratio is higher in the subendocardium. Neighboring microvascular units (with fewer numbers of parallel-running capillaries) would likely be in closer proximity in the subendocardium, due to transmural narrowing towards the endocardial surface. Thus, as before, microsphere-containing capillaries found increasingly further removed from a reference vessel might have originated from a separate microvascular unit. Finally, experimental hypertrophy due to pressure overload was not a contributing factor to the above findings. It is possible that the lack of any significant differences between control and experimental groups may, at least in part, be the consequence of the intense capillary proliferation associated with imposing the overload in such young animals. Whether similar capillary flow patterns would prevail with cardiac hypertrophy induced in adult or aged animals is unknown.

The technique of *in vivo* microsphere infusion into the left atrium of small experimental animals, however, has certain restrictions. First of all, extremely large numbers of microspheres are required to obtain an adequate number of flow vector observations, since only a small percentage actually enter the coronary circulation. Furthermore, the application of this technique is particularly difficult

in small animals. Our subsequent study discussed in Chapter 4, Part B of this dissertation, describes this technique for the comparative evaluation of capillary flow direction in adult versus developing hearts using the isolated perfused heart model. To validate this model for determining capillary flow vectors, results obtained from our *in vivo* study were used as the basis for comparison with data obtained from our *in vitro* investigation. Of particular interest was the evaluation of capillary flow direction in the younger heart, in light of previous data demonstrating significant alterations in capillary geometry during normal growth. Since both longitudinal and lateral expansion of the capillary set with growth have previously been described (Batra and Rakusan, 1992), it was reasonable to hypothesize that commensurate adaptations in capillary flow direction might be evident. More specifically, if individual capillary sets were characterized by uniform internal flow direction, then the smaller capillary sets of adjacent microvascular units in the developing heart could enhance the probability of detecting countercurrent capillary flow patterns.

The data presented first and foremost (Chapter 4) served to confirm the validity and reliability of the isolated perfused rat heart model for the estimation of capillary flow direction. Moreover, the technical advantages of this approach in the isolated heart proved particularly suitable in the case of smaller developing hearts. Overall, results were not appreciably different from those obtained from our earlier *in vivo* study. The fact that there were no significant differences between adult and young hearts may illustrate maturity of microcirculatory networks by three weeks of age. Although certain indices of capillarization may

differ markedly with normal growth, adaptations or changes in capillary flow direction are apparently not necessary to provide favorable geometric conditions for oxygen supply. Unfortunately, for technical reasons we were not able to obtain reliable results in animals prior to 21 days of age. Whether any differences in flow direction relative to older animals would be evident during this more proliferative phase of capillary growth remains, unfortunately still unanswered.

A natural extension of these studies was to consider what functional implications interventions such as acute experimental hypoxia may have on capillary flow direction. Specifically, would flow pattern alterations at the level of the coronary capillaries be a means to compensate for a significantly lowered local arterial oxygen tension? To address this question we took advantage of the technical conveniences of the isolated heart model. This study was described in Chapter 5 of this thesis. The sequential infusion of differently colored microspheres into hearts of adult male rats was employed in two series of experiments.

In the first series, a control group received three distinctly colored microsphere infusions in sequence during normoxic perfusion. This control group served to establish the "normal" capillary flow pattern based upon, for the first time, three sequential microsphere injections An "hypoxic" group received the initial two infusions under identical conditions, with the third infusion during conditions of experimental hypoxia. Hearts in an additional series of experiments received only two differently colored infusions, the initial under normoxic conditions, with the second infusion during hypoxia.

The principal finding of this investigation was the increased proportion of countercurrent capillary flow revealed subsequent to hypoxia. Histological assessment of the sequence of colors trapped within capillaries revealed a significantly higher percentage of countercurrent flow observations among neighboring capillaries following the hypoxic intervention than during normoxic perfusion conditions. These observations were reflected by a change in position or a reorientation of the final injected microspheres relative to the positions of microspheres injected prior to hypoxia.

Nevertheless, our measurements of coronary flow during the periods of microsphere infusion disclosed two strikingly dissimilar time courses. Although we appeared to have good evidence for alterations in capillary flow pattern, these experiments were not helpful in elucidating whether the initial period of high flow hypoxia or the succeeding period of diminished coronary flow (likely due to localized ischemia) was responsible for the flow reversals evidenced histologically. This limitation to our assessment was addressed through the second series of experiments employing two microsphere colors. These experiments were not hindered by the extreme reductions in coronary flow as in those experiments utilizing three microsphere colors. We considered it practicable to compare the flow vector observations from this series of experiments with the initial 2-color observations formed during normoxia in the previous series. These comparisons which revealed significantly lower proportions of concurrent flow than with the 2-color normoxic interval confirmed our hypothesis that changes in capillary flow direction were primarily the result of hypoxia itself.

Although there are certain caveats to the use of isolated heart preparations that may temper extrapolation of results to actual *in vivo* conditions, on the basis of our present findings we suggest that the direction of capillary flow is influenced by the local oxygen gradients in the myocardium. Unfortunately, an adequate explanation for alterations in capillary flow direction during hypoxia remains elusive. Nevertheless, as discussed earlier in this dissertation, an alternating countercurrent-concurrent flow pattern could conceivably provide a compensatory mechanism in an effort to provide adequate (albeit at a lowered level) oxygen delivery to local muscle cells. This would be possible since neighboring capillaries would no longer be at equivalent oxygen tensions, as predicted for purely symmetric concurrent capillary flow. We therefore interpret these results as a potential counterbalancing response at the level of the capillary network to support myocyte oxygenation resulting from a severe reduction in the arterial PO_2.

Our colored microsphere method could further be of value in the future for elucidation of unsolved questions concerning the directions of microvascular blood flow and blood supply in, for example, reperfused and infarcted myocardium. Additionally, the potential effects of various cardiomyopathies (eg. cardiomyopathic hamster, diabetic cardiomyopathy) are some other issues that may be reasonably approached in myocardium.

Our technique may be equally suitable to providing similar information on capillary flow pattern in other organs which have sufficient blood flow and capillary density. One such organ is the brain, which due to its protective

environment, as well as a prior lack of a suitable technique, has escaped accurate analysis. It would be a logical and meaningful extension of our present studies to undertake an investigation of microvascular flow directions in the brain, which due to the heterogeneity of tissue present may reveal regional flow pattern specificity.

The mammalian coronary microcirculation is an intriguing phenomenon of evolutionary design, which while dauntingly complex in organization, provides the heart with an efficient system for conducting the transport of oxygen from the red blood cells to active muscle. Using our novel experimental method, we have attempted to describe two important properties of this system for which accurate and reliable information was heretofore unavailable. The material presented in this treatise will, undoubtedly provoke further questions, for which there are no immediate answers. Future investigations will hopefully provide us with even more precise anatomical and functional descriptions into the ultimate role of the terminal vascular bed.

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