

# Research in Cardiac Surgery

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## I. Introduction

Since the first successful heart operation by Rehn (1) in 1896, cardiac surgery in adults has evolved rapidly from simple closure of heart wounds to complex repairs of congenital and acquired heart diseases (Table I). Major milestones include the ligation of the ductus arteriosus in 1937 (2–4), resection of an aortic coarctation in 1944 (5–7), closed mitral valvulotomies in 1948 (8–11), and, perhaps most importantly, development and application of cardiopulmonary bypass in the 1950s (10, 12, 13). Although coronary artery grafting as we know it today can be traced back to the early experiments by Alexis Carrel in 1910 (14), myocardial revascularization as a therapeutic modality for the treatment of ischemic heart disease began in 1967 (10, 15–17). This year also marks one of the most remarkable achievements in cardiac surgery—the first human heart transplant (18–21).

Perhaps the most significant element linking all of these advances is their foundation in research. Each of these achievements is the result of an investigative process originating in scientific inquiry into surgical problems, continuing with experimentation in laboratory models, and culminating in successful clinical cases. Furthermore, these developments represent the diligence of surgeons and researchers who continually challenged themselves to develop safer and more effective treatments for patients with life-threatening heart disease. These individuals possessed a unique element of adventure, curiosity, perseverance, and courage. Each milestone reflects their ability to apply sound scientific principles and methodologies in a laboratory setting to test hypotheses based on knowledge of cardiac physiology held at the time. Their technological developments and scientific achievements have enabled surgeons to perform more procedures on the heart with relative ease and safety. As a consequence, cardiac surgery is now one of the most commonly performed operations in the United States. Nevertheless, the challenge of surgically treating patients with heart diseases in the future is no less than it was 25 or 50 years ago.

Until recently, many of the most important advances made in cardiac surgery have been technological in nature, primarily developments allowing for new types of operations or improvements on existing procedures. Yet, despite the use of new and creative surgical techniques, patients today still die or experience major complications, requiring therapy after both closed and open-heart procedures. In

**Table I Early Developments in Cardiac Surgery Research**

Year	Development	Ref.
1882	Block sutures wounds in rabbit hearts	1
1896	Ludwig Rehn, M.D., performs first successful suture in the human heart	1
1905	Harvey Cushing, M.D., experiments with mitral valvulotomy in dogs	8
1905	Alexis Carrel, M.D., conducts early exploration into organ transplantation, involving transplantation of the heart of one dog onto the carotid artery and jugular vein of another	19
1907	John C. Munro, M.D., reports on experiments with ligation of the ductus arteriosus on cadavers of newborn infants	4
1910	Alexis Carrel, M.D., attempts revascularization of the ascending and descending aorta in dogs	14
1916	Jay McLean, M.D., discovers heparin	12
1925	Henry S. Souttar, M.D., performs the first successful mitral valvulotomy	9
1931–1953	John Gibbon, M.D., develops the heart–lung machine	10, 13
1935–1936	Clarence Crafoord, M.D., experiments with correction of aortic coarctation in dogs	5
1937	John W. Streider, M.D., performs ligation of the ductus arteriosus. Patient dies on fourth post-operative day due to acute dilation of the stomach	2
1938	Robert E. Gross, M.D., experiments with correction of aortic coarctation in dogs	6
1938	Robert E. Gross, M.D., performs successful ligation of the ductus arteriosus	3
1944	Clarence Crafoord, M.D., performs successful correction of aortic coarctation	5
1945	Robert E. Gross, M.D., performs successful correction of aortic coarctation	7
1948	Following his own research with the procedure in the 1940s, Charles P. Bailey, M.D., performs a series of mitral valvulotomies. After this point, the procedure becomes more widely used	10, 11
1950	W. G. Bigelow, M.D., reports on use of hypothermia to lengthen the period of total cardiac inflow occlusion in animal models	10
1953	Following years of experimentation in animal models, Claude S. Beck, M.D., performs successful myocardial revascularization	15
1954–1955	C. Walton Lillehei, M.D., uses controlled cross-circulation and a bubble oxygenator for intracardiac surgery in 45 patients	10
1958	Richard Lower, M.D., and Norman Shumway, M.D., begin experimentation with heart transplantation in dogs	21
1962	Vladimir Demikhov, M.D., publishes “Experimental Transplantation of Vital Organs,” reporting on his research with organ transplantation in animal models since 1950	20
1967	C. N. Barnard, M.D., performs the first human-to-human heart transplant procedure	18
1967–1969	Following his own experimentation with the procedure, as well as that of other pioneers in myocardial revascularization, W. D. Johnson, M.D., reports on the use of coronary artery bypass grafts in 301 patients, a seminal study marking the beginning of modern CABG surgery	16, 17

patients with ischemic heart disease, the mortality after first-time revascularization [coronary artery bypass grafting (CABG)] ranges between 2 and 3% for low-risk elective patients; it may be as high as 10–20% for high-risk emergent patients. The incidence of perioperative myocardial infarction continues to range between 3 and 7%. In fact, it may be higher depending on the definitions and criteria (22, 23). Finally, myocardial stunning or reversible postischemic myocardial dysfunction often occurs after all types of heart surgery, possibly in as many as 70–90% of the patients. In the setting of depressed left ventricular function, a long aortic cross-clamp time, a repeat coronary artery operation, or an emergent combined valve/CABG procedure, myocardial stunning can contribute significantly to increased morbidity and mortality in high-risk patients (24, 25).

In some respects, solutions to the major challenges facing cardiac surgeons today require less of an emphasis on

technological developments and more of an emphasis on experimentation at the basic science level. The next major advance in cardiac surgery may come from a better understanding of the mechanisms of disease in order to manipulate the physiology of the heart to minimize and/or eliminate surgery-related complications. Because surgical reparative procedures usually involve a period of ischemia/reperfusion, there is a great need to elucidate the mechanisms underlying phenomena such as stunning, hibernation, classic ischemic preconditioning, the second window of preconditioning, and pharmacologic preconditioning. We need to identify the cellular and subcellular disturbances that exist among the various sarcolemmal receptors and intercellular signaling pathways and effectors that are involved in limiting both postischemic cardiac dysfunction and myocardial necrosis. Finally, we need to know more about the mechanism(s) underlying apoptosis, or programmed cell death, and the role it plays

in the development of myocardial infarction and ventricular remodeling.

This is not to say that investigation into new technological advances that make cardiac surgery safer is not important or necessary. Surgeons and researchers cannot ignore the potential benefits, for example, of developments in robotics, minimally invasive surgical techniques, and the application of computer technology in cardiac surgery. Exploration in these areas will, and should, certainly continue. Presently, however, the main thrust of research in cardiac surgery is at the cellular, subcellular, and molecular levels. The next generation of surgical scientists will need to focus their research on the pathophysiology of myocardial disease in order to develop methods for preventing the complications resulting from cardiac surgery. The purpose of this chapter is to review some of the animal preparations and laboratory techniques that are currently being used to acquire this new knowledge.

## II. Myocardial Stunning

Myocardial ischemia is characterized by reduced ventricular function and altered myocardial metabolism. If coronary blood flow is restored within 15–20 min, the injury is reversible, but myocardial contractility may remain depressed for hours to days. This reversible postischemic ventricular contractile dysfunction that occurs despite restoration of normal blood flow is termed myocardial stunning (26, 27) (Table II). There is considerable evidence that the primary mediators of stunning include intracellular calcium overload and oxidative stress induced by reactive oxygen species (ROS) generated at the onset of reperfusion [28, 29]. Although it is known that intracellular calcium may return to normal levels early during reperfusion, transient increases in intracellular calcium can lead to increases in calcium-dependent protein kinase C and proteases, and a

concomitant reduction in myofilament calcium sensitivity (28, 30). Likewise, a release of ROS can injure intracellular proteins such as SR calcium ATPase, the ryanodine receptor, and the contractile proteins, the latter most likely being the primary targets. Regardless, although the actual mechanism of stunning is still unknown, there is considerable evidence that the interactions between calcium overload and ROS play important etiologic roles. Because myocardial stunning contributes substantially to postoperative morbidity and mortality after heart surgery, intense efforts are underway to elucidate the phenomenon's relationship to receptor activation, intracellular signaling pathways, and common effectors. This has resulted in the use of numerous experimental preparations, methods, and techniques to study the pathophysiology of stunning both *in vivo* and *in vitro*.

### A. Preparations for the Study of Myocardial Stunning

#### 1. Intact Animals

The most commonly used model to study *in vivo* myocardial stunning is a regional myocardial ischemia preparation in which a distal segment of the left anterior descending (LAD) or left circumflex (LCX) coronary artery is occluded. Such regional ischemia stunning experiments can be performed in both large (pig, dog, sheep) and small (rabbit) animal preparations. Large animal models provide the opportunity to simultaneously measure changes in ventricular function, coronary blood flow, and metabolism, in both the stunned and normally perfused myocardium, and each animal can serve as its own control. One of the primary determinants of the degree of stunning is the amount of coronary collateral blood flow during ischemia. Because dogs, but not pigs, have a well-developed native coronary collateral circulation, collateral blood flow during ischemia must be measured in all canine regional ischemia studies. Myocardial stunning can also be studied in global ischemia studies during which animals are placed on cardiopulmonary bypass

**Table II Evidence for Ischemic Preconditioning, Stunning, and Chronic Hibernation in Humans**

Ischemic Preconditioning	Stunning	Hibernation
Human atrial trabeculae	Unstable angina	Unstable and stable angina
Cultured human cardiac myocytes	Acute myocardial infarction with early reperfusion	Acute myocardial infarction
Warm-up angina	Exercise-induced ischemia	Improved LV function after angioplasty
Preinfarction angina	Open-heart surgery	Improved LV function after coronary artery bypass grafting
Coronary angioplasty	Cardiac transplantation	Congestive heart failure
Cardiopulmonary bypass		
Coronary artery bypass surgery		

and the heart is arrested with or without a cardioplegic solution. Although these studies are technically demanding, they do more closely mimic the patient undergoing heart surgery, compared to regional ischemia studies. The use of hypothermia and cardioplegia permits the ischemic period to be extended beyond the typical 15 min of ischemia used in normothermic regional ischemia studies. Regardless of which stunning model is used, it must be verified that the ischemic time utilized to induce stunning is not associated with myocardial infarction.

In the intact animal, global ventricular function is assessed by measurements of left ventricular developed pressure (LVDP), rate of change of left ventricular pressure ( $\pm dP/dt$ ), LV end-diastolic pressure (LVEDP), and cardiac output. Ventricular pressures are typically measured using a high-fidelity Millar transducer placed through the ventricular wall in the apical region. Cardiac output is measured using a volumetric ultrasonic flow probe placed around the aorta. However, these measurements are inadequate to assess regional ventricular function in regional ischemia studies and are also load dependent. Regional ventricular function is most frequently evaluated by measuring wall thickening (WT) or segment length (SL). Pairs of piezoelectric crystals are normally placed in the LAD and LCX perfused beds such that the distance between either the epicardial and endocardial walls (WT) or a segment of ventricular muscle at a certain depth (SL) is measured during systole and diastole by sonomicrometry. The resulting measurements of systolic WT (SWT) and segment shortening (SS) provide a simple, reliable measurement of regional ventricular function. These piezoelectric crystals can also be used in chronic studies.

The primary limitation to the use of measurements of wall thickening and segment shortening is their sensitivity to changes in heart rate, preload, and afterload. This limitation can be overcome by generating end-systolic pressure thickness and end-systolic pressure length relationships (ESPTR and ESPLR, respectively). Left ventricular pressure thickness/length loops are generated during acute changes in preload or afterload produced by brief ( $\sim 5$ – $7$  sec) occlusions of the vena cava or aorta, respectively. The slope of the line of ESPTR or ESPLR ( $E_{es}$ ), which is analogous to end systolic elastance ( $E_{es}$ ) of the end-systolic pressure volume relationship (ESPVR) derived from pressure volume loops, has been shown to be relatively load independent over physiologic pressure ranges. The availability of inexpensive high-speed personal computers and data acquisition software allow for real-time contractility measurements during the experiment. Data collected during the generation of pressure-segment length loops can be further analyzed to measure stroke work (SW), preload recruitable stroke work (PRSW), and preload recruitable stroke work area (PRSWA). Stroke work is afterload sensitive, but PRSW and PRSWA are sensitive and reliable indicators of cardiac contractility independent of pre-

load and afterload. Reportedly, PRSWA is the most sensitive parameter for quantifying regional ischemia-induced ventricular dysfunction (31, 32).

Load-insensitive measurements of cardiac contractility have also been used in global myocardial ischemia studies, based on LV pressure-volume relationships. One technique involves the measurement of LV pressure with a high-fidelity pressure transducer, and ventricular dimensions via the placement of epicardial dimension transducers/crystals along the major and minor axes of the ventricle. Dimension measurements are converted to volume based on the ventricle being modeled as a prolate ellipsoid. An alternative approach is the use of the conductance catheter method to measure electrical conductance of blood in the ventricle (33). Although the cost of this technology has limited its widespread use, recent developments permit the measurement of load-independent cardiac contractility in small animals such as rats and mice.

Because myocardial stunning is defined as reversible postischemic dysfunction on the restoration of coronary perfusion, coronary blood flow must be measured. For the majority of regional and global ischemia studies, the placement of an ultrasonic coronary flow probe on a short segment of dissected coronary artery will suffice. These probes are also designed for use in chronic studies. However, in certain cases, such as in canine models that can exhibit significant coronary collateral blood flow, microsphere techniques must be used to measure residual blood flow during regional ischemia. Microspheres must also be used when measuring blood flow heterogeneity or transmural distribution of blood flow (subepicardial vs. subendocardial). Although this technique has historically relied on the use of radioactive microspheres, the use of fluorescent microspheres has now become accepted.

Metabolic changes that occur in ischemic and stunned myocardium can be assessed by several means. The myocardial biopsy technique is used for obtaining tissue samples of rapidly metabolized compounds such as lactate, ATP, and creatine phosphate (CrP). Transmural tissue samples, which can often be divided into subepicardial and subendocardial layers, are rapidly collected with a standard needle biopsy technique or a rapid-freezing biopsy drill to minimize metabolism and contamination. Samples are then stored in liquid nitrogen until biochemical assays are performed. This method for monitoring myocardial metabolism works well when a limited number of samples are obtained, but multiple biopsies cause tissue trauma and deterioration of the preparation. A noninvasive technique that provides some of the same information as tissue biopsies is nuclear magnetic resonance ( $^{31}\text{P}$ -NMR) spectroscopy, although this technique is generally not feasible in large animal preparations. Coronary venous or coronary sinus blood samples can be collected to measure the release of numerous metabolites, such as lactate, adenosine, nitric oxide, norepinephrine, and reactive

oxygen species, from the area of interest. This technique can be used to supplement metabolic measurements made with tissue biopsies. When used as the sole source for metabolic measurements, however, this technique may not provide an accurate assessment of metabolites from the myocytes or those to which myocytes are exposed. For example, due to the rapid metabolism of adenosine by endothelial cells and erythrocytes, plasma adenosine measurements do not always reflect interstitial fluid levels of adenosine to which the cardiac myocytes are exposed.

A technique that has been used for years to measure neurotransmitter release in the brain, the microdialysis technique, has gained widespread acceptance for estimating interstitial fluid (ISF) metabolites in the heart (34). This technique is based on the principle that small metabolites present in the ISF can diffuse into a small, hollow dialysis fiber inserted into the ventricular muscle. A specified length of dialysis fiber, which is exposed within the muscle wall, is connected to inert silica tubing on both ends. As a physiologic saline solution passes through the fiber, diffusion occurs between the fluid within the dialysis fiber and the ISF surrounding the fiber. The concentration of a compound in the effluent or dialysate collected from the outflow tubing is representative of the intramyocardial ISF concentration of that substance. Because the ISF bathes the cardiac myocytes, the ISF level of a given compound can be used to assess myocyte release rates, substrate utilization, and the presence or absence of exogenously administered agents used to test their cardioprotective effect on stunning. Cardiac microdialysis has been used to measure numerous ISF metabolites such as adenosine, norepinephrine, lactate, and oxygen free radicals (34–37). The advantages of the microdialysis technique are the minimal setup expenses involved and the fact that this technique can provide continuous serial sampling of myocardial ISF metabolites before, during, and after ischemia. The disadvantages of the technique are that it provides only an estimate of ISF concentrations, and there is some tissue trauma incurred during implantation of the fibers.

## 2. Isolated Perfused Heart Preparations

Another model used to elucidate the detrimental effects of myocardial ischemia on ventricular function is the isolated perfused heart preparation. The standard Langendorff perfused isolated heart is based on the retrograde perfusion of the heart via the aorta and the resultant antegrade coronary perfusion. Hearts in this model can be perfused via either constant flow or constant pressure. However, because left ventricular pressure development is perfusion pressure dependent, ischemia/reperfusion and ventricular function studies in this model should be performed at constant pressure. A variation of this model is the ejecting isolated heart preparation, also referred to as the working heart, which exhibits much higher workloads and O<sub>2</sub> consumption than

the Langendorff preparation. In this model, the left atrium is cannulated and connected to a low-pressure reservoir, the aorta is cannulated and connected to a high-pressure reservoir, and the ventricle ejects against this pressure head. Left ventricular output can be altered by adjusting preload, afterload, or both.

Both the Langendorff and ejecting heart preparations are routinely perfused with a buffered physiologic salt solution, e.g., Krebs–Henseleit bicarbonate buffer. The buffer is gassed with 95% oxygen and 5% carbon dioxide to yield  $P_{O_2}$  and  $P_{CO_2}$  values of ~600 and 40 mmHg, respectively, and pH ~7.4. The buffer must be filtered through a small-pore (~0.45- $\mu$ m) filter to remove small particulate matter that can occlude the capillaries. In the majority of studies glucose (11 mM) is the sole energy source, although it is not uncommon to include pyruvate or fatty acids (the latter in the presence of albumin). Extracellular Ca<sup>2+</sup> concentration should be no greater than 2.0 mM, because free plasma Ca<sup>2+</sup> concentration is in the 1.0–1.5 mM range. Although many studies are performed with 2.5 mM Ca<sup>2+</sup>, it has been reported that this extracellular Ca<sup>2+</sup> concentration accelerates and exacerbates ischemia/reperfusion in this preparation (38). An increasing number of investigators, recognizing the limitations of a blood/protein-free perfusate, now perfuse the Langendorff preparation with erythrocyte-supplemented Krebs buffer or whole blood. This modification increases the O<sub>2</sub>-carrying capacity and viscosity of the perfusion medium, which significantly reduces the edema and high coronary flow rates observed in Krebs-perfused hearts.

Ventricular function in the nonejecting preparation is generally measured with a fluid-filled latex balloon, inserted into the left ventricle, that can be filled with varying volumes to generate LV end-diastolic pressures (LVEDP) of 5–10 mmHg. Although pressure–volume curves can be generated prior to and following ischemia, measurement of ventricular function is typically studied using an isovolumetric preparation. The use of the isovolumetric model permits the assessment of ischemia/reperfusion on diastolic function by recording changes in LVEDP. Heart rate can also be controlled by pacing the hearts throughout the study.

The simplicity and high degree of control over experimental conditions with this model permit the study of numerous aspects of ischemia/reperfusion injury on ventricular function. Studies performed in this model are typically performed in rat and rabbit hearts utilizing zero-flow global ischemia, but both low-flow ischemia and hypoxia are also easily studied. These studies are most frequently conducted in the Langendorff preparation with zero-flow global normothermic ischemia, although the ejecting heart preparation is also used. Postischemic ventricular dysfunction in the Langendorff model is assessed by recording changes in systolic and diastolic pressures and/or  $dP/dt$ . Both normothermic and hypothermic ischemia, with or without cardioplegic arrest, can be studied using this preparation.

Although there have been numerous studies on the effects of global ischemia on ventricular function in isolated perfused hearts, in the vast majority of these cases it is likely that the observed decreases in postischemic function are not due entirely to stunning. This is due to the fact that normothermic ischemia times in isolated rat heart studies are typically 25–30 min in duration, but ischemia times  $\geq 15$ –20 min are associated with cell death. Deficits in postischemic function thus could be due to stunning, necrosis, or, more likely, to a combination of the two. Based on the numerous studies in the literature, the study of true stunning in the isolated perfused rat heart is difficult, at best. Twenty minutes of global normothermic ischemia in the Krebs-perfused rat heart is associated with a significant increase in reperfusion LVEDP, which is often accompanied by little decrease in systolic LV pressure. However, stunning, as originally defined, is a reversible deficit in systolic function. This limitation needs to be considered when designing stunning experiments.

In order to verify the use of a true stunning model if the ischemic period exceeds 15 min, the extent of irreversible injury or infarction should be determined by one of two methods. The first involves the collection of coronary effluent prior to ischemia and at specific time points throughout reperfusion to measure creatine kinase (CK) release. With this approach, the reperfusion period can be limited to 45–60 min, by which point recovery of LVDP has attained its maximal value. Alternatively, the hearts can be reperused for 2 hr, after which they are stained with a triphenyl-tetrazolium chloride (TTC) solution, which stains viable myocardium brick red (due to the presence of dehydrogenase enzymes). If necrosis is to be determined by TTC staining, the hearts must be reperused for a minimum of 2 hr to obtain valid estimates of necrosis.

### 3. Other Studies

Many of the same metabolic measurements made in intact animals can be performed in isolated perfused hearts. In global ischemia studies, coronary effluent samples can be readily collected and analyzed for various substrates and catabolites. Tissue samples can be obtained for measurements of cellular metabolites, as described for *in vivo* studies, in both global and regional ischemia studies. The small sizes of rat, rabbit, guinea pig, and mouse hearts are well suited for the use of  $^{31}\text{P}$ -NMR spectroscopy. The cardiac microdialysis technique can also be used to estimate ISF metabolites. Metabolic studies with radioactive tracers are routinely used in this preparation.

### 4. Ventricular Cardiomyocytes

Isolated ventricular cardiomyocyte preparations permit the determination of whether ischemia/reperfusion exerts direct effects on cardiac myocytes. The primary advantage

of studying single cardiac myocytes is the ability to determine the role of intracellular pH,  $\text{Ca}^{2+}$  and other ions, reactive oxygen species (ROS), and other signaling pathways in cardiac myocyte injury. New imaging agents and confocal microscopy permit the measurement of intramitochondrial  $\text{Ca}^{2+}$  concentrations. The majority of such studies have been performed in ventricular myocytes obtained from rats, guinea pigs, and rabbits, although there are studies in which myocytes have been obtained from canine, porcine, and even human hearts (39, 40).

In general, myocytes are isolated using a Langendorff preparation in which the heart is digested initially with a  $\text{Ca}^{2+}$ -free physiologic salt solution (PSS), followed by the reintroduction of  $\text{Ca}^{2+}$  and the enzymes collagenase and hyaluronidase. After various other steps, a relatively homogeneous yield of ventricular myocytes is obtained (41). The quality of the myocyte yield should be verified by determining the percentage of quiescent, rod-shaped cells and/or the number of cells excluding trypan blue. Neonatal ventricular myocytes can be easily cultured, but adult myocytes are typically studied the day of isolation (primary culture). Adult myocytes also do not contract, and thus for contractility studies the myocytes must be electrically stimulated.

There have been numerous studies over the past several years on the effects of simulated ischemia/reperfusion on isolated ventricular myocytes. Although the majority of these studies have investigated mechanisms of cell death, myocardial stunning can also be studied. As in the isolated heart studies, the ischemic or hypoxic insult must be strong enough to induce a contractile deficit but devoid of cell death. Utilizing myocyte suspensions, ischemia is simulated by centrifuging an aliquot of cells into a small pellet and removing the majority of the suspension medium/buffer. The pellet is then covered with a thin layer of mineral oil to prevent myocyte exposure to atmospheric  $\text{O}_2$ . The packed myocyte pellet in the reduced extracellular volume mimics ischemic conditions. These ischemic incubations are typically performed in the absence of glucose or other energy substrates, and the medium may contain elevated lactate and  $\text{K}^+$  to simulate changes that occur during *in vivo* ischemia. After 15–20 min of simulated ischemia, the myocytes are resuspended in an oxygenated medium to simulate reperfusion. Myocyte contractility is measured with a video edge detection system to determine the extent and rate of shortening.

In single myocyte studies, standard fluorescent imaging techniques/systems and various cell-permeable fluorescent dyes permit the measurement of intracellular ions during simulated ischemia/reperfusion. A video edge detection system coupled to the imaging system provides the ability to correlate changes in myocyte contractility with alterations in intracellular metabolites/ions. Myocyte stunning in single cells can be induced with mild hypoxia/reoxygenation or low-flow ischemia.

### III. Hibernating Myocardium

Reversible contractile dysfunction that corresponds to or matches a reduction in resting coronary artery blood flow is termed “hibernating myocardium.” It represents a balanced reduction in myocardial contraction and myocardial oxygen consumption (42). In general, it is found in patients with severe coronary artery disease and is associated with stable and unstable angina, myocardial infarction, and congestive heart failure. Because it is fully reversible on restoration of normal blood flow, in humans the phenomenon can only be inferred from the findings in clinical studies (43–53) (Table II). Interestingly, human tissue biopsies obtained from hibernating myocardium have shown a dedifferentiation of myocardial cells with the loss of myofibrils, glycogen accumulation, and variable degrees of interstitial fibrosis (42, 54–56). Imaging techniques used to assess hibernating myocardium in humans include the use of positron emission tomography (PET) scanning, thallium redistribution scintigraphy, and dobutamine stress echocardiography. These studies are used to identify the subset of patients with low ejection fractions who would benefit from myocardial revascularization via percutaneous coronary interventions or coronary bypass surgery.

Considerable controversy persists, however, whether the contractile dysfunction is due to an absolute reduction in coronary blood flow or to a reduction in coronary reserve. Likewise, there is controversy whether myocardial hibernation and myocardial stunning may coexist or share a common mechanism (57). Various characteristics of these two phenomena are listed in Table III. It is also unclear whether chronic contractile dysfunction in humans reflects repetitive episodes of stunning or an intrinsic adaptation to ischemia. The latter implies that the heart can alter its phenotype to protect itself from irreversible ischemic injury (52, 58).

Consequently, it is not surprising that a number of various animal models have been developed to investigate the perfusion-matching adaptation process. Although the isolated perfused hearts have and can be used to study hibernation, these findings have to be interpreted in the context of denervation, use of crystalloid buffer perfusates, the use of global rather than regional ischemia, and the generally chronic nature of myocardial hibernation. Also, isolated hearts demonstrate little metabolic recovery during prolonged ischemia in contrast to metabolic studies performed *in situ* (43, 59). As a consequence, short-term hibernation is most frequently studied in chronically instrumented dogs, pigs, or miniature swine subjected to regional ischemia. In these short-term hibernation preparations, accumulating evidence suggests that adaptation does occur. If the mechanisms can be elucidated, it may be possible to develop therapeutic strategies that will allow the heart to adapt much more rapidly to ischemia and thus avoid permanent injury (myocardial necrosis) (43, 57).

#### A. Short-Term Hibernation Models

In the chronically instrumented model described by Sherman *et al.* (60), dogs are sedated and then subjected to general anesthesia. Under sterile conditions, a left thoracotomy is performed in the fifth intercostal space to expose the heart. The dogs are then instrumented for chronic measurements of cardiac and systemic hemodynamics, similar to the parameters described earlier in the *in vivo* myocardial stunning preparation. Following 7 to 10 days of recovery from surgery, the dogs, under light sedation, are subjected to 2 hr of sustained reductions in regional flow sufficient to cause approximately a 50% reduction in regional systolic segment shortening or wall thickening. The heart rate can be maintained constant by atrial pacing to minimize changes in

**Table III Characteristics of Reversible Postischemic Myocardial Dysfunction**

Stunning	Hibernation
Dysfunctional myocardium with normal or near-normal blood flow	Dysfunctional myocardium with reduced blood flow
Contractile abnormality reversible with time	Contractile abnormality reversible on reperfusion
Absence of irreversible damage	Absence of irreversible damage
Perfusion imaging (PET scan) normal or increased	Perfusion imaging (PET scan) increased
Contractile function decreased	Contractile function decreased
No metabolic deterioration during inotropic stimulation	Recruitment of inotropic reserve is at the expense of metabolic recovery
Disruption of myofibrillar structure in canine model	Disruption of myofibrillar structure in canine model
Heart does not adapt to chronic underperfusion	Heart adapts to chronic underperfusion
Steady state between perfusion and contraction not achieved	Steady state between perfusion and contraction can be reached
Perfusion–contraction mismatching lasts from hours to days to months	Perfusion–contraction matching can be maintained for prolonged periods of time
Lack of evidence for dedifferentiation process in myocytes	Evidence for dedifferentiation process in myocytes

hemodynamics. Measurements are performed before and during the period of reduced blood flow. Depending on the experimental design, the animals can be euthanized at the end of the 2 hr of reduced flow or at a predetermined time point after reperfusion (hours to days). In these studies, measurements of regional myocardial blood flow with microspheres, myocardial oxygen consumption, and regional ventricular function are critical to verify the presence of hibernating myocardium. Measurements of tissue metabolites with biopsies or sampling of the affected bed's coronary venous blood can also be taken to assess metabolic changes.

One disadvantage of this acute preparation is that it deals primarily with short-term changes in hibernating myocardium that, by definition, may not be directly translatable to humans with chronic (months to years) hibernation. The model does, however, meet the traditional definition of hibernating myocardium, i.e., proportionate reductions in systolic function and resting coronary blood flow. More importantly, the use of chronically instrumented conscious dogs or pigs avoids the numerous problems associated with isolated hearts and nonsurvival surgery. It is important to recognize, however, that even in the conscious state, changes in the activity of the animal can lead to an imbalance between myocardial oxygen supply and demand, resulting in a degree of stunning.

### B. Long-Term Hibernation Models

In order to study long-term hibernation in animals, a technique described by Fallavollita *et al.* can be utilized to provoke coronary collateral growth in pigs (61, 62). In this model, anesthetized pigs undergo a left lateral thoracotomy in the fourth intercostal space to expose the heart and the proximal LAD coronary artery. The artery is mobilized for a short distance and then instrumented with a 5-mm Delran occluder with a fixed internal diameter ranging between 1.5 and 2.2 mm. After securing the artery within the occluder with a silk ligature, the chest incision is closed. Approximately 3–4 months later, again under general anesthesia, the animals are returned to the laboratory for the performance of hemodynamic studies and procurement of tissue for analysis. To document regional perfusion under resting conditions, colored microspheres can be injected into the left ventricle followed by contrast left ventriculography and selective coronary angiography (63). After completion of the angiography the animals are euthanized and the heart is examined for molecular alterations. Characteristically, this model is associated with depressed anteroapical wall motion, severe proximal stenoses, or total LAD occlusion and angiographic evidence of collaterals.

Regardless of how well the various animal preparations meet the criteria of hibernation, it is important to recognize that all animal experiments are limited by a fixed observa-

tion time. In real terms, myocardial hibernation is a clinical phenomenon of contractile dysfunction that exists in patients with coronary artery disease. Although it may exist for months or years, it is fully reversible on reperfusion (64).

## IV. Ischemic Preconditioning

A brief period of sublethal ischemia that reduces irreversible tissue injury secondary to a period of subsequent prolonged ischemia is termed ischemic preconditioning (IPC). The attendant reduction in infarct size is associated with a reduced rate of myocardial high-energy phosphate and glycogen catabolism, and reduced accumulation of lactate, hydrogen ions, and catecholamines (65–67). Ischemic preconditioning, which has also been shown to be very effective in reducing ventricular tachycardia and fibrillation, has been shown to occur in every species studied to date, including humans (68–70) (Table II), and in multiple organ systems in addition to the heart. Although the specific triggers and mediators of IPC have not been unequivocally determined, current evidence suggests likely roles for adenosine, nitric oxide, and reactive oxygen species (71). In terms of intracellular signaling mechanisms, the primary focus has been on protein kinase C (PKC), mitogen-activated protein kinases (MAPK), and ATP-dependent K<sup>+</sup> channels (72).

In 1993, Marber *et al.* and Kuzuya *et al.* independently reported that IPC induces a biphasic pattern of protection against myocardial necrosis (73, 74). This form of protection, termed delayed preconditioning or the second window of preconditioning (SWOP), has been confirmed in the rat, rabbit, dog, and pig, including both open-chest and chronically instrumented conscious animals (75–80). Thermal, metabolic, hypoxic, and anoxic delayed preconditioning have also been reported to be protective in cultured rat and rabbit cardiac myocytes (81–86). Unlike acute or classical IPC, however, SWOP requires >6 hr to develop, the protection is conferred around 24 hr later, and it may persist up to 72 hr. It is usually manifested as a reduction in both myocardial stunning and infarct size, and is associated with a decrease in ventricular arrhythmias (73, 79, 87).

In the course of determining the triggers and mechanisms of acute and delayed IPC, it became apparent that preconditioning's protective effects could be mimicked by numerous pharmacologic agents. These agents include, but are not limited to, adenosine and adenosine A<sub>1</sub> and A<sub>3</sub> receptor agonists (88–93), ATP-dependent potassium channel openers, bradykinin, and nitric oxide donors (94–96). This phenomenon is referred to as pharmacologic preconditioning because the heart is typically exposed to a brief drug infusion that is terminated 10–15 min prior to the onset of ischemia. This treatment is distinct from a pretreatment protocol in which the pharmacologic agent is not administered until the onset

of ischemia. The ability to pharmacologically precondition the heart may be most applicable to cardiac surgery. In such a scenario, patients could be administered a pharmacologic treatment 24 hr and 5–10 min prior to cross-clamping the aorta during open-heart surgery. The development of pharmacologic preconditioning agents that not only mimic IPC but extend the duration of protection could markedly improve or replace currently employed intraoperative myocardial preservation techniques. Lists of pharmacologic agents used to mimic IPC and those used to inhibit IPC are included in Tables IV and V.

Although all forms of preconditioning are protective against infarction, there are differences with regard to the stunned myocardium. *In vivo* canine and porcine studies indicate that acute ischemic preconditioning does not attenuate myocardial stunning (97, 98). In contrast, delayed preconditioning or SWOP does appear to attenuate stunning in conscious porcine and rabbit models (99, 100). There also appear to be differences in the effects of pharmacologic pre-

conditioning on stunning and infarction. For example, although adenosine preconditioning (in which the infusion is terminated prior to ischemia) reduces infarct size in the rabbit, it does not reduce stunning (101, 102).

## A. Preparations for Studying Preconditioning

### 1. Intact Animals

Ischemic preconditioning has been studied in a variety of intact animals, including rats, rabbits, dogs, pigs, and sheep. These usually involve regional ischemia protocols in which regional ischemia is achieved by tightening a snare (or an occluder in the case of a chronic experiment) to occlude the distal portion of the left anterior descending coronary artery. Five minutes of complete occlusion is usually followed by 5 min of reperfusion before 30–60 min of regional ischemia (depending on the species) and 180 min of reperfusion. Several reports have indicated that *in vivo* myocardium must be reperfused for a minimum of 180 min to provide a reliable

**Table IV Tools Used to Study Ischemic Preconditioning: Agents That Mimic Classic and Delayed IPC<sup>a</sup>**

Agent	Proposed action
Adenosine (ADO)	ADO A <sub>1</sub> , A <sub>3</sub> receptor agonist (classic IPC)
Cyclohexyl adenosine (CHA)	ADO A <sub>1</sub> receptor agonist (classic IPC)
Phenylisopropyl adenosine (PIA)	ADO A <sub>1</sub> receptor agonist (classic IPC)
Chlorocyclopentyladenosine (CCPA)	ADO A <sub>1</sub> receptor agonist (classic IPC)
Phorbol 12-myristate 13-acetate (PMA)	Direct PKC activator
Diacylglycerol (DAG)	Direct PKC activator
Dioctanoyl- <i>sn</i> -glycerol (DOG)	Direct PKC activator
Phenylephrine	α <sub>1</sub> -Adrenergic receptor agonist and PKC activator
Angiotensin II	AT <sub>1</sub> receptor agonist and PKC activator
Reactive oxygen species (ROS)	PKC activator and phospholipase D stimulator
Bradykinin	Tyrosine kinase, PKC, and NOS activator
Bimakalim	K <sub>ATP</sub> channel opener
Pinacidil	K <sub>ATP</sub> channel opener
Nicorandil	K <sub>ATP</sub> channel opener
Cromakalim	K <sub>ATP</sub> channel opener
Aprikalim	K <sub>ATP</sub> channel opener
Diazoxide	K <sub>ATP</sub> channel opener
Monophosphoryl lipid A (MLA)	K <sub>ATP</sub> channel opener
Deltorphan	Opioid receptor agonist
DADLE	Opioid receptor agonist
Nitroglycerine	NO donor (delayed IPC)
Diethylenetriamine/NO (DETA-NO)	NO donor (delayed IPC)
S-Nitroso-N-penicillamine (SNAP)	NO donor (delayed IPC)

<sup>a</sup>The effects of these agents may vary depending on species studied, experimental design, drug concentration at the site of action, and receptor selectivity. For example, many of these agents can activate more than one pathway depending on the dose used.

**Table V Tools Used to Study Ischemic Preconditioning: Agents that Inhibit Classic and Delayed IPC<sup>a</sup>**

Agent	Proposed action
Sulfophenyltheophylline (SPT)	Nonspecific ADO antagonist (classic IPC)
8-Cyclopentyl-1, 3 dipropylxanthine(DPCPX)	ADO A <sub>1</sub> receptor antagonist (classic IPC)
Staurosporine (STAU)	PKC blocker
Bisindolylmaleimide (BIS)	PKC blocker
Chelerythrine	PKC blocker
Polymyxin B	PKC blocker
Calphostin C	PKC blocker
Cycloheximide	Protein synthase inhibitor (delayed IPC)
N <sup>ω</sup> -Nitro-L-arginine (L-NA)	NO-synthase blockers (delayed IPC)
N-Nitro-L-arginine methyl ester (L-NAME)	NO-synthase blockers (delayed IPC)
Aminoguanidine (AG)	Inducible NO-synthase blockers (delayed IPC)
S-methylisothiouria (SMT)	Inducible NO-synthase blockers (delayed IPC)
Genistein	Tyrosine kinase inhibitor
5-Hydroxy decanoate (5-HD)	Mitochondrial K <sub>ATP</sub> channel blocker
Glibenclamide	Sarcolemmal and mitochondrial K <sub>ATP</sub> channel blocker
Naltrexone	Opioid receptor antagonist
HOE 140	Bradykinin receptor blocker

<sup>a</sup>The effects of these agents may vary depending on species studied, experimental design, drug concentration at the site of action, and receptor selectivity. Without measuring the activity of the target directly, one must be cautious in interpreting the findings. For example, BIS, a PKC blocker, has been reported to exert a cardioprotective effect under certain conditions (130). BIS and STAU have been reported to not inhibit IPC in the dog and pig (44). Glibenclamide and 5-HD have been reported to have variable results in the rat (131). Furthermore, many of these agents can inhibit more than one pathway, depending on the dose used.

estimate of infarct size. Regional ventricular function can be measured as described earlier for stunning experiments, but in the majority of *in vivo* preconditioning studies this is not done, because the primary objective of IPC experiments is to demonstrate a reduction in infarct size.

As in regional stunning experiments, the area of infarct size and the area at risk (AAR) or ischemic bed size must be measured. Infarct size is then expressed as a percentage of the AAR. The AAR can be determined by perfusing the aortic root with monastral blue dye after ligation of the involved artery at the end of the experiment. The area of the left ventricle supplied by the occluded coronary artery (ischemic bed) is identified by the absence of the dye. In small animals, fluorescent microspheres can also be used to determine AAR. The heart is then cut from the base to the apex into four to five slices, the thickness of which is determined by the particular species being studied. The most frequently used method to differentiate viable from infarcted myocardium is a process whereby the slices are incubated in a 1% solution of triphenyltetrazolium chloride at 37°C for ~ 20 min. The slices are then weighed, photographed, and fixed in a 1% formaldehyde solution. Transparencies are then projected at a 10-fold magnification and traced to delineate nonischemic, ischemic/reperfused, and infarcted regions. The corresponding areas are then measured by

videoplanimetry and from these measurements infarct size can be calculated as a percentage of the ischemic bed.

Three key factors must be carefully controlled during *in vivo* animal studies when measuring infarct size (and stunning). Although monitoring and maintenance of normothermic body temperature is often taken for granted, it has been reported that small (<2°C) decreases in body temperature can significantly reduce both infarct size and stunning. Infarct size is also directly dependent on the size of the area at risk. For this reason, it is not always sufficient to merely report infarct size as a percentage of the area at risk, but also as infarct volume. This can be accomplished easily by cutting the heart slices to an exact thickness so that the volume of infarct and risk areas can be determined. Finally, as mentioned earlier, coronary collateral blood flow must be measured in canine regional ischemia studies, because the degree of ischemic injury correlates with the amount of residual collateral flow during ischemia.

Intact animal preparations are also commonly used for delayed ischemic and pharmacologic preconditioning studies. The majority of these studies have been conducted in rabbits and rats, due to the reduced expenses and ease in handling smaller animals, although several studies of this type have been performed in pigs (99, 103). In SWOP ischemic preconditioning infarction protocols, the animals

are initially instrumented with a suture or balloon occluder under open-chest, sterile conditions and then allowed up to a week to recover from the surgery prior to performing the preconditioning protocol. In delayed pharmacologic preconditioning studies, the drug being investigated can be administered (IP or IV) in a sedated or anesthetized closed-chest animal. After the preconditioning intervention, the animals are returned to their cages until the prolonged occlusion experiment is performed under open-chest conditions 24–72 hr later. To exclude the effects of anesthesia and the stress of open-chest conditions on the results obtained in intact animal preconditioning studies, conscious rabbit models have been developed (104). Procedures similar to those described above are used, with the exception that the prolonged ischemia is also conducted in conscious animals. Following the prolonged occlusion, the hearts can be reperfused for several days prior to determining myocardial infarct size.

Studies of delayed preconditioning effects on stunning have almost without exception been performed in conscious animals. In these studies, the animal is initially instrumented under sterile conditions with not only a coronary occluder device, but also with either a pulsed doppler ultrasonic epicardial crystal or segment-shortening crystals in the area at risk to measure regional ventricular function. Initial studies of this phenomenon were performed in conscious pigs (103), but subsequent studies have been performed in conscious rabbits (99). Delayed preconditioning is induced by subjecting the heart to six 4-min occlusions interspersed with 4 min of reperfusion. Successful occlusion is verified by changes on the electrocardiogram and the appearance of myocardial wall thinning on the crystal recordings. Regional myocardial function (stunning) is assessed using the doppler probe to measure systolic wall thickening (STw) after each of the brief occlusions and up to 5 hr after the sixth reperfusion. These findings are then compared to the similar measurements obtained after repeating the protocol on days 2 and 3. Improvement in the recovery of STw after the six ischemia/reperfusion cycles are repeated on days 2 and 3 indicates that delayed preconditioning is effective in reducing postischemic stunning. When this preparation is used it is important to demonstrate the absence of infarction using the tissue-staining TTC technique described earlier. This will confirm that the injury associated with the ischemia/reperfusion cycles resulted in reversible injury.

## 2. Isolated Hearts

Isolated heart models are also frequently used to study delayed preconditioning effects on infarct size. The majority of these experiments have involved pharmacologic preconditioning studies in which the animals, typically rabbits, are treated with the agent being studied 24–72 hr prior to excising the heart. The isolated perfused heart is then submitted to a regional ischemia protocol in which infarct size is measured. To date, there have been relatively few studies

in which the effects of delayed preconditioning on ventricular function have been studied in the globally ischemic isolated heart.

## 3. Cardiomyocytes

The use of ventricular cardiomyocytes (neonatal and adult rats and adult rabbits) facilitates the determination of mechanisms of injury and protection by preconditioning at the myocyte level (84, 85, 105). Myocyte cell suspensions are submitted to simulated ischemia/reperfusion as described earlier for the myocyte stunning model, with the exception that the ischemic period is longer (30 min to 3 hr, depending on the species). Ischemia-induced cell death in aliquots of myocytes is determined by measuring CK or LDH release into the medium or by the percentage of cells that do not exclude trypan blue. A viability curve is then calculated (time versus cell death), and the area under the curve is used to facilitate the comparison of IPC protection with the protection provided by various agents used to mimic or block preconditioning. Preconditioning is induced with 10 minutes of pelleting in glucose-free medium and 15 min of resuspension in oxygenated buffer to most closely mimic IPC. Alternatively, myocytes can be preconditioned with hypoxia or anoxia. Study drugs are selected on the basis of their ability to inhibit or enhance selectively potential mediators of necrosis. To simulate reperfusion, the myocytes can be resuspended in standard oxygenated buffer containing  $\text{Ca}^{2+}$ . This myocyte suspension model is well suited for the determination of the effects of ischemia and reperfusion and cardioprotective treatments on cardiac myocytes. However, reversible and irreversible injury to cell membranes during ischemia and/or reperfusion and intracellular acidosis may preclude the ability to correlate injury with changes in intracellular ions determined with fluorescent indicators (81, 86, 105, 106).

Although myocyte preparations are best suited for studying the acute effects of preconditioning, they can also be used for delayed preconditioning studies. This is most easily accomplished in myocytes that culture well, such as neonatal rat or fetal chick myocytes (107). However, these cells are typically more resistant to ischemia than are adult myocytes, so longer ischemia times must be used to induce cell death. In addition, results obtained in chick myocytes should be verified in a mammalian species. At least one group has cultured adult rat ventricular myocytes for 24 hr to study delayed preconditioning (83, 85).

## 4. Human Tissue

The assumption in all experimental animal models is that preconditioning's beneficial effects also apply to humans. This has led to the establishment of several models of preconditioning using human tissue, such as cardiomyocytes and atrial trabeculae obtained from humans at the time of surgery, most frequently tissue obtained from the right atrial

cannulation site in patients undergoing coronary artery bypass surgery (68). Studies involving the use of human atrial trabeculae have investigated the effects of IPC on contractility and cell death (70, 89). In the former, one end of the trabecula is attached to a fixed post in an organ bath and the other is attached to a force transducer to measure force generation of stimulated muscles. The muscle is then suspended in an organ bath superfused with an oxygenated physiologic salt solution. Ischemia is simulated with prolonged hypoxic substrate-free superfusion and rapid pacing followed by reperfusion with normal oxygenated buffer. Preconditioning is induced by exposing the trabecula to 3 min of hypoxic substrate-free superfusion with rapid pacing (3 Hz) followed by 7–10 min of reperfusion with oxygenated buffer. Differences in contractile function are assessed after prolonged simulated ischemia. Cell death in these same protocols, or in experiments specifically studying necrosis, is based on measurements of enzyme release (CK, LDH, or troponin I) into the medium.

Although the above studies permit determination of the protective effects of preconditioning on cardiomyocytes, similar observations must be documented in whole heart and/or by *in vivo* studies to verify their physiological relevance. This obviously cannot be done in the case of human tissue when the endpoints are contractility and infarction. Additional limitations of preconditioning studies in human tissue include (1) the limited amount of available tissue, (2) the difference between atrial tissue and myocytes and ventricular myocytes, and (3) the source of ventricular myocytes (when available, they are obtained from diseased hearts, which likely exhibit altered physiology and signaling mechanisms).

Regardless of the model of IPC utilized or species studied, there is the underlying assumption that limiting infarct size is desirable, i.e., a reduction in myocardial necrosis correlates with improved postischemic function. Experimentally, this is not always demonstrable. For example, although it is well established that IPC in the globally ischemic isolated perfused rat heart improves postischemic function, this is generally not the case in the perfused rabbit heart (108,

109). There are some reports, however, that improved functional recovery does correlate with a reduction in infarct size in *in vivo* regional ischemia preparations (109–112). Thus, the investigator needs to take into account not only the species but also the preparation when trying to elucidate the mechanism(s) and effects of preconditioning on infarct size and function.

## V. Apoptosis

Apoptosis, or programmed cell death, is now recognized as a possible cause of noninflammatory cardiac myocyte loss and thus cardiac muscle dysfunction in numerous conditions, such as ischemia/reperfusion injury, remodeling, heart failure, hypertrophy, and aging (113–115). Etiologic mechanisms include reperfusion following ischemia, acidosis, ventricular pressure overload, oxidative stress, and activation of several cytokines and receptor agonists (116). Clinically, the phenomenon has been reported in humans with long QT syndrome, hibernation, acute myocardial infarction, and chronic heart failure (117).

Because it is one of the two phenomena responsible for myocyte loss, the characteristics of apoptotic death have been morphologically and biochemically compared to cell death from necrosis (Table VI). Necrosis, or accidental cell death, is associated with significant decreases in energy stores, with swelling, and eventually with rupture of sarcolemmal, mitochondrial, and nuclear membranes and with nuclear chromatin clumping. In contrast, the apoptotic cell, which exhibits near normal energy levels, shrinks, membranes and intracellular organelles remain intact, and the nucleus condenses and breaks into nucleosomes and the DNA fragments. Another major difference between the two causes of cardiomyocyte loss is the difference in the manner in which the cells are removed. Necrotic cells are removed by an inflammatory process, whereas apoptotic cells are phagocytosed by neighboring cells.

Although the exact mechanisms underlying apoptosis are unknown, there is increasing evidence that reactive oxygen

**Table VI Myocyte Loss after Ischemia/Reperfusion**

Necrosis	Apoptosis
Myocyte swells and ruptures	Myocyte shrinks
Nuclear chromatin clumps into dense masses	Nuclear chromatin condenses and fragments
Nucleus swells	Nucleus condenses and fragments into small nuclear bodies
Mitochondria undergo swelling; cristae fragment and accumulate insoluble calcium-containing phospholipid complexes	Mitochondria and cell membrane remain intact
Dead myocytes removed by inflammatory process	Focal noninflammatory destruction of myocytes
Cytosolic accumulation of calcium and sodium ions	Active, gene-directed process; caspases play a crucial role

species plays an important role. This is based on observations in myocardial ischemia/reperfusion studies that apoptosis commences primarily during reperfusion concomitant with the formation of intracellular ROS and/or intracellular calcium overload (28, 118). This programmed cell death appears to be initiated by the translocation of the proapoptotic proteins Bad and Bax from the cytosol to the mitochondrial membrane. Heterodimerization of Bad or Bax with the antiapoptotic Bcl-2-xl leads to the release of the mitochondrially localized cytochrome *c* into the cytosol (119–121). Formation of a cytosolic complex consisting of cytochrome *c*, apoptosis activating factor-1 (APAF-1), and caspase 9 leads to activation of caspase 3 and the cleavage of poly(ADP)-ribosylating (PARP) protein (36). Activation of PARP appears to be the final step in apoptosis leading to DNA fragmentation (122). The production of ROS and/or intracellular calcium overload also induces mitochondrial permeability transition pore (MPTP) opening (123), which, if not reversed, can result in the loss of mitochondrial proteins and ions.

For years myocardial ischemia/reperfusion injury was generally studied in terms of reversible postischemic dysfunction (stunning and/or hibernation), which progresses to irreversible injury or necrosis with prolonged ischemia times. However, although considerable debate has continued as to whether reperfusion injury truly occurs, it has not been fully understood why a substantial portion of myocardium within the ischemic zone does not show signs of necrosis. With the recognition of the phenomenon of programmed cell death, it now appears that a portion of these cells, which otherwise appear to be normal based on gross morphology, may be undergoing apoptosis. Furthermore, cells in the early stages of apoptosis that are unable to maintain normal oxidative phosphorylation in the face of oxidative stress and decreased antioxidant reserves may eventually die via necrosis. By its very nature, apoptosis, or programmed cell death, may be much more amenable to effective treatments than is accidental or necrotic cell death.

Myocardial apoptosis can be studied in the same *in vivo*, isolated heart, and in isolated myocyte preparations previously described (62, 115, 124–126). Because current evidence suggests that myocardial stunning is associated with minimal apoptosis (114), ischemia times should exceed 20 min to induce programmed cell death. At the conclusion of the *in vivo* and isolated heart regional ischemia experiments the hearts are stained to determine the area at risk and the infarct size. Following this, one or more tissue samples are harvested and frozen in liquid N<sub>2</sub> or in an ultracold freezer (−80°C) prior to preparing specimens for apoptosis assays.

Three techniques are currently utilized to assess apoptosis in intact myocardial tissue: (1) DNA fragmentation via terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL assay), (2) DNA laddering using SDS gel electrophoresis (SDS-PAGE), and (3) electron micro-

scopy. Many investigators use at least two different methods for the identification of apoptosis in a given experiment to minimize false-positive results. For example, Lim *et al.* demonstrated the presence of apoptosis in an *in vivo* porcine model of hibernating myocardium by TUNEL and electron microscopy (62). Although TUNEL and DNA fragmentation provide qualitative findings, if combined with other techniques the extent of myocyte apoptosis can be quantitated. Thin slices of fixed tissue sections can be appropriately stained and examined under high-magnification light microscopy to demarcate cell membranes and differentiate myocytes from other cell types. The TUNEL assay in combination with epifluorescence permits quantitation of the number of fluorescing nuclei of myocyte origin confirmed by examination at high power (600×). The extent of apoptosis is expressed by normalizing the results to the number of myocyte nuclei per square millimeter.

Electron microscopy can be used to differentiate necrosis from apoptosis. The latter cells have nuclear chromatin margination and condensation in the presence of intact sarcolemmal and mitochondrial membranes. Fresh tissue samples are fixed in glutaraldehyde, postfixed in osmium, and embedded in Embed Araldite mixture using routine procedures. Ultrathin sections are stained with uranyl acetate and lead citrate.

Measurement of apoptosis in intact hearts is typically determined by assessing DNA fragmentation using the TUNEL assay and DNA laddering. The former is most frequently detected using the APOP TAG kit (Oncor, Gaithersburg, MD) in which free 3′-OH DNA ends, generated by DNA fragmentation and typically located in nuclei and apoptotic bodies, are labeled by TdT (an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA) (127). The principle of the method is catalytic addition of residues of digoxigenin-labeled nucleotide to the DNA by TdT. Antidigoxigenin antibody conjugated to peroxidase is then used with chromagen substrate to generate an immunohistochemical signal of the presence of DNA fragmentation, indicating apoptosis.

Apoptosis is also verified by the cleavage of DNA into nucleosomal fragments in multiples of 180 base pairs, which form a laddering pattern on polyacrylamide gels. For DNA laddering, sections are fixed with 10% formalin, embedded in paraffin, sliced into 5-μm sections, and stained with hematoxylin–eosin and trichrome for light microscopy examination. Extraction of DNA is performed using a nonorganic DNA extraction kit (e.g., Oncor). Fresh tissue is minced in phosphate-buffered saline and incubated at 50°C for 3–6 hr with occasional shaking in lysis buffer containing proteinase K and DNase-free RNase A. The DNA solution is then extracted with phenol, precipitated in ethanol, and resuspended in buffer. For analysis of DNA fragmentation, DNA is fractionated by electrophoresis on a 1% agarose gel.

The DNA is visualized by ethidium bromide staining and exposure to ultraviolet light.

Apoptosis in cardiac myocyte preparations can be measured by the TUNEL assay and DNA laddering. There are two advantages to studying apoptosis in isolated cardiac myocytes. One advantage is that because the myocytes are studied in isolation, there is no need to distinguish myocytes from other cell types. A second advantage is the ability to detect apoptosis at a much earlier stage than is detected by the TUNEL assay and DNA laddering. There is evidence that one of the earliest signs of apoptosis is the translocation of phosphatidylserine from the inner surface of the plasma membrane to the cell surface (128, 129). These residues are then accessible to fluorescein isothiocyanate (FITC)-conjugated annexin V, which is a member of a family of proteins that has a strong affinity for phosphatidylserine. Because annexin V fluoresces a bright green when bound to phosphatidylserine on the outer surface of apoptotic cells, this stain can be used to identify early programmed cell death. Viable cells, when excited at 488 nm, fluoresce at 520 nm; however, this fluorescence is of low intensity and can be distinguished from the bright intense fluorescence of FITC-labeled annexin V apoptotic cells.

Apoptotic cells can be distinguished from necrotic cells by the differential staining pattern observed with nuclear dyes such as propidium iodide and Hoechst 33342. Propidium iodide binds to DNA, but does not cross intact cell membranes; thus, it will stain only the nuclei of necrotic cells or those with leaky membranes. When excited at 488 nm, propidium iodide fluorescence is detectable as intense red fluorescence. This method of measuring cell death is thus similar to that determined by using trypan blue exclusion. The Hoechst stain, which is membrane permeable, labels all nuclei; however, under high magnification apoptotic cells exhibit fragmentation of chromatin, degradation of the nuclear envelope, and nuclear blebbing, resulting in the formation of condensed nuclei (micronuclei). Because necrotic cells can also stain positive with FITC-labeled annexin V, cells that stain positive for both dyes are considered necrotic, whereas myocytes that stain positive only for annexin are considered apoptotic.

These staining assays are very simple, because they involve only incubation of aliquots of myocytes or single cells with annexin V and propidium iodide or Hoechst 33342, and visual examination of the cells on an imaging system using the appropriate fluorescent filter combinations. In single-cell studies, it may be possible to measure intracellular  $\text{Ca}^{2+}$ , ROS, or other metabolites if the dyes used fluoresce at a different wavelength than that of FITC-labeled annexin V. Cell suspension studies are more feasible, and with the use of flow cytometry, cell death in a large sample of cells can be rapidly and reliably determined.

Although the procedures described above determine the presence of apoptotic cell death, they do not provide any

information on the mechanisms of this form of cell death. Thus, a complete examination of apoptosis should include measurements of one or more various steps in this program of cell death. These include, but are not limited to, the translocation of cytochrome *c* from the mitochondria to the cytosol, the activation of caspase 3, measurements of the pro- and antiapoptotic proteins Bad/Bax and Bcl-2, and even the intracellular reduced and oxidized glutathione levels, the key metabolite in the cell's antioxidant defenses. It is thought that all of these proteins and metabolites are altered in either their concentration and/or subcellular location prior to the development of DNA fragmentation or laddering. Establishing the time frame for these changes in relation to the development of cell death may be clinically relevant. For example, in current ischemia/reperfusion studies the presence of apoptosis is based on measurements of DNA fragmentation and laddering, which are the final steps in apoptotic cell death. Even the efficacy of antiapoptosis treatments, such as caspase inhibitors, has been based primarily on reduction of infarct size, not reduction of apoptosis. However, once the nucleus is damaged and DNA is fragmented, the cell's ability to synthesize new repair proteins is severely compromised, and damaged cells, even if they survive a first ischemic episode, may die at an accelerated rate during subsequent stress or ischemia. If cell death in ischemic/reperfused myocardium progresses from apoptosis to necrosis, and if the program of cell death in early apoptosis can be interrupted and even possibly reversed, then the most beneficial treatments would be those targeted to early events in apoptosis.

## VI. Conclusion

Although research in cardiac surgery has changed dramatically over the past 50 years, the problems facing cardiac surgeons today are just as challenging as those solved by clinician-scientists in the past. This is due to the increasing need to develop and implement therapies to treat the complications that occur after open-heart surgery. The aim is to not only improve survival, but also to enhance the overall quality of life after heart surgery. Research in cardiac surgery, however, is and must continue to be grounded in inquiry at the basic science level. Although cardiac transplantation, replacement of heart valves, performance of complex congenital heart disease operations, and coronary artery bypass grafting represent spectacular accomplishments, fundamentally all these corrective procedures still involve exposing the heart or a region of the heart to a period of ischemia. As a consequence, few, if any, cardiac operations are completely free of significant complications. Heart transplantation is a good example of a procedure in which postischemic myocardial dysfunction plays an important role in outcome. The 30-day mortality after cardiac replacement currently

ranges between 5 and 15% and is most frequently related to primary graft dysfunction secondary to prolonged ischemic times and/or inadequate myocardial preservation. Allograft rejection, donor heart ischemia, progressive myocyte loss over time, and the development of graft vasculopathy also have been associated with prolonged ischemic times. Regardless of the cause, the need to improve both the short- and long-term survival is urgent, especially because the 1- and 5-year survival rates after cardiac transplantation still remain at 80 and 60%, respectively. Likewise, although cardiac surgeons can successfully replace heart valves, repair complex congenital heart defects, and revascularize the severely ischemic heart, these operations do not necessarily prevent patients from experiencing myocyte loss over time, which ultimately can progress to heart failure. Thus, it is not surprising that myocardial stunning, myocardial hibernation, ischemic preconditioning, and apoptosis are currently areas of major investigation in cardiac surgery today.

More investigation is needed into myocardial stunning and hibernation in order to comprehend fully the events that take place during both ischemia and reperfusion. Furthermore, research is needed to elucidate how the purported primary mediators, intracellular calcium overload, and oxidative stress caused by ROS generated at the onset of reperfusion result in reversible and irreversible myocardial injury. More specifically, experiments need to be designed to determine how derangements in the various proposed targets of ROS, such as the sarcolemma, the sarcoplasmic reticulum, the mitochondria, and the extracellular collagen matrix, actually result in stunning and hibernation. Likewise, studies are needed to address the role ischemia/reperfusion plays in modulating intracellular acidosis and activation of the sodium/hydrogen exchanger. If the latter is responsible for increased transsarcolemmal calcium influx and intracellular overload, is the evidence strong enough to warrant the use of  $\text{Na}^+/\text{H}^+$  exchange inhibitors to prevent or reduce the incidence of myocardial infarction or death after open-heart operations? What are the roles, if any, of gene regulation and receptor expression in stunning and hibernation? What are the reparative processes that take place during the recovery process and why does recovery time vary according to the duration and degree of ischemia? When does reversible injury become programmed irreversible injury? Does apoptosis represent just one aspect of a continuum of ischemia, and, if so, is programmed cell death reversible, and at what point in time?

Similarly, there are numerous other questions that need to be answered with respect to both classic and delayed preconditioning. Future research must focus on the triggers, mediators, intracellular signaling pathways, and effectors that actually modulate this powerful endogenous form of myocardial protection. Findings from these types of studies could lead to the development of new pharmacologic preconditioning agents that could be used during cardiac surgery to enhance the heart's tolerance to ischemia and reduce

the incidence of stunning, infarction, and heart failure. Currently, several endogenous and exogenous agents are under investigation as potential cardioprotective agents. These include preconditioning mimetics such as adenosine  $\text{A}_1$  and  $\text{A}_3$  receptor agonists, ATP-dependent potassium channel openers, bradykinin, and nitric oxide donors. Ultimately, elucidation of the mechanisms underlying the potent endogenous cardioprotective effects of IPC and hibernation and the deleterious consequences of stunning and apoptosis depends on focused basic science research that is innovative, hypothesis driven, utilizes modern investigative technology, and is translational in nature.

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