Dietary omega-3 fatty acids, nutritional preconditioning of the heart against ischemic injury

Grace Gulbahar Abdukeyum
University of Wollongong

DIETARY OMEGA-3 FATTY ACIDS, NUTRITIONAL PRECONDITIONING OF THE HEART AGAINST ISCHEMIC INJURY

This thesis is presented in fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

From

UNIVERSITY OF WOLLONGONG

by

GRACE GULBAHAR ABDUKEYUM, BSc, MSc in Medicine

SCHOOL OF HEALTH SCIENCES

2010

CERTIFICATION

I, Grace Gulbahar Abdukeyum, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the school of health sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Grace Gulbahar Abdukeyum

May, 2010

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank you for University of Wollongong for awarding me International Postgraduate Scholarship and University Postgraduate Award. Receiving these scholarships helped me to reduce my financial burdens. I am indebted to my supervisor Peter McLennan for giving me an opportunity to pursue PhD in his lab, patiently guiding me with my PhD project, specifically assisting me with my data analysis and interpretation of the data. Special thanks are due to my co-supervisor Stephen Lillioja, who was appointed at the last year of my PhD study, for his critical and timely comments throughout the writing of this thesis; to Alice Owen for her assistance in measuring oxidative markers and antioxidants; to Halmurat Gopur, the chancellor of Xinjiang Medical University, China, for his patience allowing me to complete my study beyond our four-year contract.

Also, I owe a great debt to my parents and my sister who never gave up on me during this long and seemingly interminable journey producing this thesis. Their encouragement is constant -even they are thousands of miles away, most of all, I would like to thank the God for putting a bright lamp on my feet and pulled me up while I stumbled through my study. Finally, thank you also to my advisory committee for their positive constructive feedback.

TABLE OF CONTENTS

GLOSSARY OF TERMINOLOGY

Acute coronary occlusion: The partial or complete obstruction of blood flow in a coronary artery.

Afterload: Resistance to ventricular ejection: measured clinically with aortic blood pressure and calculation of systemic vascular resistance.

Angina: Chest pain or discomfort that occurs when an area of heart muscle does not get enough oxygen-rich blood.

Apoptosis: Occurrence of internucleosomal fragmentation of genomic DNA associated with a sealed plasma membrane.

Bradyarrhythmias: Slow heart rate, an arrhythmia caused by failure of impulse formation or by failure of impulse conduction.

Cardiac arrest: The sudden complete loss of cardiac output and therefore blood pressure.

Cardiac Output: Cardiac output is the volume of blood being pumped by the heart, in particular by a ventricle in a minute. Cardiac output is equal to the stroke volume multiplied by the heart rate .

Cardiac arrhythmia (also dysrhythmia): Abnormal electrical activity in the heart. The heartbeat may be fast or slow, and may be regular or irregular.

Coronary Artery Bypass Graft Surgery: Colloquially heart bypass or bypass surgery is a surgical procedure where arteries or veins from elsewhere in the patient's body are grafted to the coronary arteries to bypass atherosclerotic

narrowing and improve the blood supply to the coronary circulation supplying the myocardium.

Cost-effectiveness ratios: A ratio of the change in costs of a therapeutic intervention to the change in effects of the intervention.

Diastolic arterial pressure: The lowest pressure (at the resting phase of the cardiac cycle). Typical values for a resting, healthy adult human are approximately 120mmHg systolic and 80mmHg diastolic with large individual variations.

End-diastolic pressure: The pressure in the ventricles at the end of diastole, usually measured in the left ventricle as an approximation of the end-diastolic volume, or preload.

End-diastolic volume: The volume of blood in a ventricle at the end of filling (diastole). Because greater end diastolic volumes cause greater distention of the ventricle, end diastolic volume is often used synonymously with preload.

Fibrin: Insoluble protein that is produced in response to bleeding and is the major component of the blood clot.

Free radicals: Any atomic or molecular species capable of independent existence that contains one or more unpaired electrons in one of its molecular orbitals.

Ischemia: Oxygen deprivation accompanied by inadequate removal of metabolites due to reduced perfusion. Prolonged ischemia results in tissue damage and death because of a lack of oxygen and nutrients.

Hemostasis: Refers to the physiologic process whereby bleeding is halted, thus protecting the integrity of the vascular system after tissue injury.

Ischemia reperfusion: The restoration of blood flow to an area that had previously experienced deficient blood flow.

Ischemic heart disease: A condition in which there is an inadequate supply of blood and oxygen to a portion of the myocardium; it typically occurs when there is an imbalance between myocardial oxygen supply and demand.

Ischemic reperfusion injury: Accelerated and additional myocardial injury beyond that generated by ischemia alone. It includes hastening of the necrotic process of irreversibly injured myocytes, cell swelling, the no-reflow phenomenon, hemorrhagic myocardial infarction, the calcium and oxygen paradox, the production of oxygen-derive free radicals which may damage ischemic myocytes, and the prolonged postischemic depression of ventricular function and electrophysiologic changes which in turn can cause arrhythmias.

Ischemic preconditioning: A technique in which tissue is rendered resistant to the deleterious effects of prolonged ischemia and reperfusion by prior exposure to brief, repeated periods of vascular occlusion.

Myocardial infarction (MI): A loss of cardiac myocytes (necrosis) caused by prolonged ischemia.

Myocardial stunning: The mechanical dysfunction that persists after reperfusion, despite the absence of irreversible damage and restoration of normal coronary flow.

Necrosis: The ultimate form of cell death and defined as accidental collapse of cellular homeostasis, compartmentalisation and cell membrane integrity with the release of cytosolic material and with random nuclear DNA fragmentation.

Nutritional preconditioning: Inducing preconditioning by nutritional means.

(n−3) fatty acids: n−3 fatty acids (popularly referred to as omega-3 fatty acids or ω−3 fatty acids) are a family of unsaturated fatty acids that have in common their

first carbon–carbon double bond as the third bond from the methyl end of the fatty acid.

(n−6) fatty acids: n−6 fatty acids (popularly referred to as omega-6 fatty acids or ω−6 fatty acids) are a family of unsaturated fatty acids which have in common their first carbon–carbon double bond as the sixth bond from the methyl end of the fatty acid.

Oncosis: Early plasma membrane rupture and disruption of cellular organelles, including mitochondria.

Percutaneous transluminal coronary angioplasty: Commonly known as coronary angioplasty or simply angioplasty, a form of catheter-based therapy as an alternative to bypass surgery is to treat the stenotic coronary arteries of the heart found in coronary heart disease. Angioplasty is less invasive than coronary artery bypass surgery.

Plasminogen: The inactive precursor of plasmin, a potent serine protease involved in the dissolution of fibrin blood clots.

Primary prevention: Can include prevention of atherosclerosis, acute coronary occlusion myocardial infarction and sudden cardiac death.

Pharmacological preconditioning: Inducing preconditioning by pharmacological means.

Preload: The pressure stretching the ventricular wall of the heart after passive filling and atrial contraction. Preload is theoretically most accurately described as the initial stretching of a single cardiac myocyte prior to contraction.

Secondary prevention: Prevention of fatal arrhythmias or new ischemic episodes in those who have had a prior heart attack.

Stroke volume: The volume of blood pumped out of one ventricle of the heart in a single beat.

Stroke work: The work done by the ventricle to eject a volume of blood (i.e., stroke volume) into the aorta.

Stunning: Viable myocardium, which is not contracting properly but is not acutely ischemic.

Systolic arterial pressure: The peak pressure in the arteries, which occurs near the beginning of the cardiac cycle.

Sudden cardiac death: Natural death from cardiac causes, heralded by abrupt loss of consciousness within one hour of the onset of acute symptoms; preexisting heart symptoms may have been known to be present, but the time and mode of death are unexpected.

Thrombus or **blood clot**: The final product of the blood coagulation step in hemostasis.

Thrombosis: The formation of a clot or thrombus inside a blood vessel, which obstructs the flow of blood through the circulatory system.

Ventricular tachycardia: Three or more consecutive ventricular premature beats. If persistent, causes loss of blood pressure and death.

Ventricular fibrillation: A rapid, chaotic, and asynchronous contraction of the left ventricle.

ABSTRACT

Cardiovascular disease now ranks as the leading cause of death, resulting in one third of all deaths in the world, among which ischemic heart disease is projected to be the number one cause of death globally. It has been long suggested that (n-3) polyunsaturated fatty acids provide cardiovascular protection, with regular intake of fish or fish oil associated with reduced mortality from heart diseases in both clinical trials and epidemiological studies. One major observation in these studies is that fish oil has been found to reduce mortality during or following ischemic events. While ischemia damages the heart, short bursts of ischemia paradoxically protect the heart from the damaging effects of more prolonged ischemia. This powerful cardioprotective phenomenon is termed ischemic preconditioning. The protective influences of ischemic preconditioning include reduction of infarct size, prevention of life-threatening arrhythmias in ischemia and reperfusion, reduced myocardial oxygen demand, and improved recovery of post-ischemic cardiac pumping function. However, the promise of ischemic preconditioning has not yet been realized in bench to bedside application. The characteristics of cardioprotection afforded by feeding (n-3) PUFA suggests preconditioning-like effects, related to incorporation of (n-3) PUFA into heart membranes. However, the long chain highly unsaturated (n-3) PUFA in the membranes are highly susceptible to peroxidation, perhaps making cardiac membranes more susceptible to free radical generation and cellular damage. While free radicals are thought to be involved in the damaging

effects of ischemia and reperfusion, they also play a role in protective mechanisms of ischemic preconditioning.

This study evaluated the effects of fish oil on (n-3) PUFA incorporation into myocardial membrane and examined the susceptibility to oxidative damage and myocardial injury in terms of infarct size and postischemic cardiac function. Further, it compared dietary fish oil with ischemic preconditioning and assessed their interaction for effects on heart function and injury during myocardial ischemia and reperfusion. It tested the hypothesis that the susceptibility to peroxidation may provide an ever-present preconditioning stimulus that protects the heart against the damaging effects of a major ischemia reperfusion insult.

Male Wistar rats were fed one of three fully fabricated diets containing 10% fat by weight varying only in the types of fat. The (n-3) PUFA diet contained 7% fish oil + 3% olive oil; The (n-6) PUFA diet contained 5% sunflower seed oil + 5% olive oil; The saturated fatty acid (SF) diet contained 7% saturated fat-rich beef tallow + 3% olive oil. Heart function was examined after six weeks feeding using Langendorff-perfused isolated isovolumic heart preparation. In control experiments, isolated perfused hearts were subjected to 30 minutes regional ischemia by occluding the left anterior descending coronary artery, then reperfused for 120 minutes. Ischemic preconditioning consisted of three cycles of five minutes global ischemia before the 30 minutes regional ischemia and 120 minutes reperfusion. Heart function was assessed during perfusion by ECG and by measurement of intraventricular pressure. Infarct size was measured at completion

of perfusion in control and ischemic preconditioned hearts as a percent of the ischemic zone at risk. Lipid peroxidation products and antioxidant concentrations were measured in normoxic heart and in ischemic and non-ischemic regions of hearts with or without ischemic preconditioning.

Control (n-3) PUFA hearts had significantly lower spontaneous heart rate, coronary flow, end diastolic pressure, maximum relaxation rate, and fewer ischemic reperfusion arrhythmias than did (n-6) PUFA hearts or SF hearts. In reperfusion (n-3) PUFA hearts maintained greater developed pressure and maximum rate of relaxation and developed smaller infarcts (10.9±3.6% ischemic zone, n=6) than (n-6) PUFA hearts (47.4±2.3%, n=6) or SF hearts (50.3±4.3%, n=6).

Ischemic preconditioning significantly improved heart function and reduced infarct size in (n-6) PUFA hearts (11.8±5.4%, n=6) and SF hearts (13.1±4.2%, n=6). Heart function and infarct size did not differ between control and ischemic preconditioned hearts (9.6±4.2%) with (n-3) PUFA diet. Arrhythmias were significantly reduced by ischemic preconditioning in (n-6) PUFA hearts or saturated fatty acid hearts towards levels observed in (n-3) PUFA hearts.

Myocardial membranes showed high incorporation of long chain docosahexaenoic acid (DHA) (22:6,n-3), predicting increased risk of peroxidation. The concentration of lipid hydroperoxides and malondialdehyde were higher in normoxic and nonischemic regions of control (n-3) PUFA hearts than in (n-6) PUFA or SF hearts. The concentration of the endogenous antioxidant superoxide dismutase was higher

in normoxic and nonischemic regions of control (n-3) PUFA hearts and was increased after ischemic preconditioning in saturated fatty acid and (n-6) PUFA hearts. Both (n-3) PUFA diet and ischemic preconditioning inhibited the ischemia-induced rise in the oxidation products lipid hydroperoxides and malondialdehyde.

This thesis demonstrated that the harmful effects of myocardial ischemia and reperfusion, such as infarct size, poor relaxation, cardiac arrhythmia and poor recovery of contractile function were largely curtailed by feeding an (n-3) PUFA rich diet. The effects of SF and (n-6) PUFA diet on heart function and ischemia susceptibility were largely indistinguishable, indicating that the effects of fish oil were specifically related to its (n-3) PUFA content and not due to either a reduction in saturated fat intake or a non-specific role of polyunsaturated fatty acids. Regular consumption of dietary fish oil induced sustained changes in membrane fatty acid composition and produced cardioprotection that appears similar to late ischemic preconditioning. The continuous presence of (n-3) PUFA in myocardial membranes suggests that, as reported for late ischemic preconditioning, this is not subject to desensitization.

Dietary treatment of rats with (n-3) PUFA caused an increase in peroxidation index suggesting an increase in susceptibility of the membrane to oxidative damage, which might be expected to enhance ischemic damage. An increase in membrane lipid peroxidation was indeed observed in fish oil treated rat hearts, however, it was associated with increased antioxidant activity and reduced lipid oxidation under stress and instead of causing lasting damage to heart function, beneficial effects on

arrhythmia, contractile function, and myocardial infarct size were observed. These protective effects are demonstrated as powerful as ischemic preconditioning.

Therefore, in light of cardioprotective effects of (n-3) PUFA to reduce the consequences of ischemic events in the human population when a regular part of the diet, the present thesis demonstrated that (n-3) PUFA induces a form of preconditioning in the heart, which this thesis has termed, nutritional preconditioning. The (n-3) PUFA limit ischemic cardiac injury and myocardial infarction and endow cardioprotection as powerful as ischemic preconditioning under these experimental conditions. Nutritional preconditioning by membrane incorporation of (n-3) PUFA may underpin the low cardiovascular morbidity and mortality associated with regular fish and fish oil consumption.

PUBLICATION ARISING FROM THIS THESIS

Abdukeyum, G.G., Owen, A.J., and McLennan, P.L. (2008), 'Dietary (n-3) long-chain polyunsaturated fatty acids inhibit ischemia and reperfusion arrhythmias and infarction in rat heart not enhanced by ischemic preconditioning', *J. Nutr.,* 138 (10), 1902-09.

Abdukeyum, G.G., Owen, A.J., and McLennan, P.L. (2008), 'Effect of ischemic preconditioning and fish oil on oxidative status in isolated rat hearts' (Pending submission for publication*).*

PUBLISHED ABSTRACTS PRESENTED AT INTERNATIONAL MEETINGS

Abdukeyum, G.G. and McLennan, P.L. (2007), 'Dietary fish oil mimics the cardio-protective effect of ischemic preconditioning in isolated rat heart', *FASEB J.,* 21 (6), A1223-c-.

McLennan, P.L. and Abdukeyum, G.G. (2008), 'Nutritional preconditioning of the rat heart by dietary fish oil', *Cardiovasc. Drugs Ther.,* 22 (2), 141-42.

PUBLICATION FROM OTHER WORK DURING CANDIDATURE

Dietrich, M., Hu, Y.Q., Block, G., Olano, E., Packer, L., Morrow, J.D., Hudes, M., Abdukeyum, G.G., et al. (2005), 'Associations between apolipoprotein e genotype and circulating F-2-isoprostane levels in humans', *Lipids,* 40 (4), 329-34.

CHAPTER 1

INTRODUCTION

1.1 STATEMENT OF PROBLEM AND SETTING THE SCENE

Cardiovascular disease now ranks as the leading cause of death, resulting in one third of all deaths in the world (Mackay, J. & Mensah, G.A. 2004), whereas before 1900, infectious disease and malnutrition were the most common causes of death, and cardiovascular disease was responsible for <10% of all deaths. An estimated 17.5 million people died from cardiovascular disease in 2005, and by 2015, nearly 20 million people will die from cardiovascular disease, among which acute coronary occlusion is projected to be the number one cause of death globally (Murray, C.J. & Lopez, A.D. 1997; WHO Feb. 2007). Acute coronary occlusion is defined as the sudden partial or complete obstruction of blood flow in a coronary artery. Coronary arteries carry blood containing oxygen and other nutrients essential to the normal functioning of the heart muscle. Sudden occlusion of an artery interrupts the blood supply, resulting in ischemia. Ischemia typically occurs when there is a mismatch of blood supply (oxygen delivery) and blood request (oxygen supply) for adequate oxygenation of tissue (Elliott M. Antman *et al.* 2008). If sustained, this is what is known as heart attack and if left untreated for a

sufficient period, it will cause death of the area of muscle served by that artery, which is called myocardial infarction.

The prognosis in patients is inversely associated with the extent of myocardial infarction, that is, the amount of necrotic myocardial tissue (Downey, J.M. & Cohen, M.V. 2006; Sobel, B.E. *et al.* 1972). Because the dead contractile myocytes are not compensated by division of surviving myocytes, the prevention of cell death is of utmost importance in maintaining the contractile state of the myocardium (Downey, J.M. & Cohen, M.V. 2006). It is known that immediate restoration of blood flow (reperfusion), is important in successfully salvaging ischemic tissue. However, cellular damage and dysfunction occurs during reestablishment of blood flow, and possibly lead to lethal arrhythmias and/or the death of myocardial cells that were weakened prior to the initiation of reflow (Forman, M.B. *et al.* 1990; Jennings, R.B. *et al.* 1960). Myocardial infarction is a term that reflects death of cardiac myocytes (necrosis) caused by prolonged ischemia. If myocardial infarction develops, the myocardium changes its shape, size and function as an adaptive process in response to stress. These adaptations include the formation of scar tissue and fibrosis in infarct zones through proliferation of myofibroblasts, increased deposition of extracellular matrix components and pathological hypertrophy. These deleterious alterations lead to increased stiffness, contractile dysfunction, reduced cardiac output, diastolic dysfunction, ventricular fibrillation and ventricular failure; consequently, these may contribute to ventricular dilatation, heart failure and sudden death (Burke, A. & Renu, V. 2008) .

Heart attack deaths, however, most often occur even before ischemia progresses to necrosis and infarction. The majority of deaths from a heart attack are sudden and individually unpredictable, with almost half occurring before the patient reaches the hospital and before the heart muscle has died (Fox, C.S. *et al.* 2004; Salomaa, V. *et al.* 2003). What is perhaps even more disturbing is that about two thirds of cardiac deaths occur without prior recognition of the presence of cardiac disease (Myerburg *et al.* 1993).

The term "sudden cardiac death" refers to "natural death from cardiac causes, heralded by abrupt loss of consciousness within one hour of the onset of acute symptoms; preexisting heart symptoms may have been known to be present, but the time and mode of death are unexpected" (Myerburg & Robert, J. 2005). It is reported that in approximately three quarters of cases of sudden cardiac death, ventricular tachycardia, and fibrillation (a rapid, chaotic, and asynchronous contraction of the left ventricle) are the immediate causes (Meissner, M.D. *et al.* 1991; Schaffer, W.A. & Cobb, L.A. 1975; Wilber, D.J. *et al.* 1988). Seventy five to eighty percent of the first recorded rhythm in patients presenting with a sudden cardiovascular collapse is ventricular fibrillation. Therefore, aside from preventing ischemic episodes, preventing or limiting ventricular tachycardia, and fibrillation and salvaging as much myocardium as possible during the acute phase of coronary occlusion is of utmost importance in prevention of sudden cardiac death.

Strategies for preventing sudden cardiac death are categorized as primary or secondary (Law, M.R. *et al.* 2002). Primary prevention refers to the attempt to prevent the first heart attack. An existing myocardial infarction is a known risk

factor for high incidence of fatal heart attack in the subsequent years (Law, M.R. *et al.* 2002; Wannamethee, G. *et al.* 1995). Secondary prevention refers to prevention of fatal arrhythmias or new ischemic episodes in those who have had a prior heart attack. The primary prevention strategies currently used depend upon magnitude of risk among the various population subgroups. The most powerful modifiable long-term risk factors include lifestyle and dietary habits, elevated serum cholesterol, markers of inflammation (for example, C-reactive protein concentrations), diabetes mellitus, elevated blood pressure, left ventricular hypertrophy, and non-specific electrocardiographic abnormalities (Elliott M. Antman *et al.* 2008). It has been suggested that patients screened as having a high risk should be advised on risk reduction, and to have antihypertensive or lipid lowering therapy based on the assessment of their total cardiovascular risk (Elliott M. Antman *et al.* 2008). A review of interventions to prevent coronary heart disease risk found that nutrition interventions had the most favourable cost-effectiveness of AU $724-$1949 spent per life-year gained, compared with antihypertensive medications at AU $3772 and the cholesterol-lowering medication simvastatin at AU $20585 (Ebrahim, S. *et al.* 1999). The statins are the predominant drug class used for cholesterol-lowering and typically constitute the single largest expenditure on pharmaceuticals in the world. In Australia, $806 million was spent on statins from December 2002 to November 2003 (HIC 2004). More recent studies highlight the poor economic performance of statins unless well targeted for those at high risk, with estimated cost-effectiveness ratios (a ratio of the change in costs of a therapeutic intervention to the change in effects of the intervention) of up to AU $75134 for primary prevention in men (Schectman, G. *et al.* 1993; Simons, L.A. *et al.* 2000). Long-term clinical trials of

statins have reported discontinuation rates of approximately 30 percent (Schectman, G. *et al.* 1993; Simons, L.A. *et al.* 2000). Similarly, long-term compliance with antihypertensive medication regimens has been poor. In one study, only 49% of patients took more than 80% of their prescribed dosages during the first year of treatment (Feldman, R. *et al.* 1998). Furthermore, other studies indicate that 16% to 50% of hypertensive patients quit taking their medications within the first year of treatment (Alderman, M.H. *et al.* 1996; Cohen, J.S. 2001; Feldman, R. *et al.* 1998; Tomlinson, B. 1996). The high frequency of discontinuation of antihyperlipidemic and antihypertensive drugs is largely due to adverse effects and therapeutic ineffectiveness of the drug (Andrade, S.E. *et al.* 1995; Cohen, J.S. 2001; Langsjoen, P.H. *et al.* 2005). Therefore, developing safe, low-cost interventions that can be applied to the population at large is important in preventing sudden cardiac death caused by arrhythmias. Such interventions are also important for preventing myocardial infarction and its consequences.

Experimental, clinical and epidemiological studies, and randomized trials, clearly demonstrated that foods rich in omega-3 fatty acids, most commonly fish, can provide significant protection against heart disease mortality and reduce the risk of sudden cardiac death. These range from human intervention studies (Burr, M.L. *et al.* 1989b; Harper, C.R. & Jacobson, T.A. 2005; Jacobson, T.A. 2006; Marchioli, R. *et al.* 2001; Marchioli *et al.* 2002; Mozaffarian, D. *et al.* 2005b) that show prevention of fatal arrhythmias without reducing incidence of ischemic events, to animal studies that directly show protection against fatal arrhythmias, ischemic reperfusion injury and enhanced early post ischemic recovery of ventricular

function and cardiac output (McLennan, P.L. *et al.* 1988; Pepe & McLennan, P.L. 1996; Pepe, S. & McLennan, P.L. 2002).

An alternative approach for cardioprotection is the phenomenon that has become known as ischemic preconditioning, which was first reported in 1986 by Murry and his colleagues. It was described as "a technique in which tissue is rendered resistant to the deleterious effects of prolonged ischemia and reperfusion by prior exposure to brief, repeated periods of vascular occlusion" (Murry, C.E. *et al.* 1986). The protective influences of ischemic preconditioning include reduction of infarct size, prevention of life-threatening arrhythmias in ischemia and reperfusion, reduced myocardial oxygen demand, and improved recovery of post-ischemic cardiac function (Cohen, M.V. *et al.* 1991; Shiki, K. & Hearse, D.J. 1987; Tanoue, Y. *et al.* 2002; Yellon, D.M. & Downey, J.M. 2003). A complex intracellular signalling cascade has been identified (Otani, H. 2008; Yellon, D.M. & Downey, J.M. 2003). Studies have focussed on identifying agents that might stimulate or mimic steps in the ischemic preconditioning intracellular signalling cascade. However, most pharmacological agents lack specificity to the heart. The potential side effects of ischemic preconditioning agents are not negligible. The adenosine receptor agonists as preconditioning mimetic agents produce substantial hemodynamic effects which limit the widespread application of these agents in clinical settings (Shryock, J.C. *et al.* 1998). The opioid agonists used in clinical medicine include morphine, levorphanol, meperidine, fentanyl, and methadone. They are widely used for the treatment of pain. However, the US Federal Drug Administration has not approved these drugs for use in patients with unstable angina or who have existing

myocardial infarction. This is likely due to the high potential for dependence and respiratory depression of this drug (Fryer, R.M. *et al.* 2002).

Even if a potential therapeutic agent is found, the schedule of administration is crucial for the success of a cardioprotective drug. The preconditioning treatment must be administered before the onset of ischemia. Unfortunately, it is rarely possible to predict an impending ischemic event that might lead to acute myocardial infarction. This limits the possible clinical application of ischemic preconditioning or its mimetic agents.

In summary, despite advances in identifying pharmacological approaches to mimic ischemic preconditioning, their lack of cardiac specificity and potential side effects largely limit their use in clinical settings. Neither brief episodes of ischemia nor pharmacologic agents that mimic ischemia are routinely used as therapy in patients in hospitals (Kloner, R.A. 2006; Kloner, R.A. & Rezkalla, S.H. 2006).

Ischemic preconditioning is known to have two distinct phases. An early phase (early preconditioning) that is protective for only a few hours and a late or delayed phase, which develops after 12 hours and lasts for 72-96 hours (Bolli, R. 2000). Early preconditioning is subject to desensitisation with early experimental evidence suggesting that the protective effects are lost after prolonged periods of repetitive ischemia (Cohen, M.V. *et al.* 1994). Developing tolerance to ischemic preconditioning mimetic agent might also be a problem for long-term therapy with those agents based on mechanisms of early preconditioning. On the other hand,

delayed preconditioning is not desensitized and lasts for 72-96 hours. Preconditioning stimuli need to be re-applied during this time period to maintain protective effects (Bolli, R. 2000).

Based on the clinical evidence of cardio protective effects of habitual fish consumption and fish oil, together with specific experimental evidence of effects on heart function, the thesis hypothesises that (n-3) PUFA may provide a form of preconditioning that could explain its cardioprotective effects. The thesis sought to answer the question: "Could (n-3) PUFA provide cardioprotection similar to preconditioning?" It was assumed that a dietary approach with a safe and effective nutritional component would overcome the need to predict the onset of ischemic episodes and as a regular component of the diet, it would provide long term cardioprotection. Indeed, epidemiological studies showing long-term fish consumption is associated with reduced cardiovascular disease mortality suggest this (Mozaffarian, D. 2008).

1.1.1 Outline of chapters

Chapter One -Introduction

Chapter Two broadly describes the current knowledge of cardioprotective effects of ischemic preconditioning and the cardiovascular health benefits of (n-3) PUFA and the possible mechanisms of ischemic preconditioning. It also describes the parallel effects of (n-3) PUFA membrane enrichment and preconditioning, and outlines the thesis question.

Chapter Three describes the common methods used in this study. It begins with a rationale for choosing diet and ischemic preconditioning protocols. Detailed description of diet preparation, general experimental procedures and data acquisition are stated. Preliminary data are illustrated to show the stability of the experiments.

Chapter Four describes effects of dietary fat on heart membrane fatty acid composition, comparing saturated fat diet, (n-6) PUFA diet and (n-3) PUFA diet.

Chapter Five examines key aspects of haemodynamic function of hearts, including heart rate, measures of contraction and relaxation as well as recovery of function, cardiac arrhythmia and heart muscle damage (infarct size) after ischemia.

Chapter Six covers the role of oxidation and antioxidants markers in the mechanism of (n-3) PUFA and ischemic preconditioning.

Chapter Seven discusses the results of the experimental chapters in relation to each other and the literature and makes final conclusion.

CHAPTER 2

LITERATURE REVIEW

2.1 (N-3) PUFA AND HEART DISEASE

The American Heart Association (Kris-Etherton, P.M. *et al.* 2002), the European Society for Cardiology (Backer, G.D. *et al.* 2003) and other professional health agencies including NHF Australia and NHMRC Australia have issued recommendations for increased intakes of (n-3) PUFA, which are found in fish and fish oils. These recommendations are based on evidence derived from a variety of scientific approaches linking low dietary intake of long chain (n-3) PUFA with increased risk for cardiovascular events, especially sudden death (Harris, W.S. *et al.* 2006a; Jacobson, T.A. 2006; Psota, T.L. *et al.* 2006; Reiffel, J.A. & McDonald, A. 2006; Robinson, J.G. & Stone, N.J. 2006; Wang, C. *et al.* 2006).

The most consistent effects of (n-3) PUFA on the cardiovascular realm have been antiarrhythmic, antithrombotic, hypolipidemic and small, dose dependent effects on blood pressure. While all these factors are related to cardiovascular risk, only the antiarrhythmic effects can be demonstrated at intakes consistent with reduced cardiovascular disease mortality. The antiarrhythmic effects are also the only of these effects associated with the incorporation of (n-3) PUFA into the heart itself. The following review describes the role of fats in the heart and is focused on the

outcomes of (n-3) PUFA in epidemiological and interventional studies that describe the cardio protective effects (n-3) PUFA on cardiovascular disease in patients with pre-existing cardiovascular disease, as well as in healthy individuals and animal studies.

2.1.1 What are essential PUFA?

Fats are the most concentrated form of energy for the body. They also aid in the absorption of the fat-soluble vitamins, A, D, E and K and other fat-soluble biologically-active components. Chemically, most of the fats in foods are triglycerides, made up of a unit of glycerol combined with three fatty acids, each of which may be the same or different. Other dietary fats include phospholipids, phytosterols and lipoproteins associated with cholesterol. The different types of fatty acids are the most important characteristic of dietary fats.

According to the degree of unsaturation (double bonds and hydrogen content), fatty acids are largely classified into three major types: saturated fatty acids, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). A fourth form, the trans fatty acids, are mainly produced by partial hydrogenation of polyunsaturated oils in food processing but also occur naturally in animal foods in small amounts.

Fatty acids consist of a hydrocarbon chain with a hydrophobic methyl group at one end and a hydrophilic carboxyl group at the other end. The Greek alphabets (α, β, γ, ω) have been used to identify the location of the double bonds in fatty acids. The

"alpha" carbon is the carbon closest to the carboxyl group. The methyl end of the molecule is also referred to as the omega end, and the terminal carboxyl group is located at the delta end. Current chemical numeric literature numbers the carbon chain from one to “n” with n being the last carbon at the methyl end. The terms “n” and “omega” are synonymous.

Saturated fatty acids contain no double bond; they are fully saturated with hydrogen. The main saturated fatty acids are lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) (Cx is the number of carbons, see Figure 2.1). Saturated fats are found mainly in animal-based products, such as milk, cream, butter and cheese, meats from most land animals, palm oil and coconut oil, as well as manufactured products made from these, such as pies, biscuits, cakes and pastries.

The main monounsaturated fatty acid in the human diet is oleic acid with one double bond (C18:1). Monounsaturated fatty acids are predominant in plant-based foods, such as olive oil, canola oil, sunola oil and peanut oil, and are also found in high proportions in animal fats. Polyunsaturated fatty acids contain two or more chemical double bonds between adjacent carbon atoms and are identified according to their chain length, number of double bonds and the position of the bonds. Based on location of the first double bond (starting from the methyl group, or omega end, the tails), PUFA can be divided into two main families; (n-3) PUFA and (n-6) PUFA. The most common PUFA is linoleic acid (LA; C18:2n-6), the parent fatty acid of (n-6) PUFA class (Figure 2.1).

Linoleic acid is found in large amounts in western diets in seed oils, such as corn oil, safflower oil, sunflower oil, and soybean oil. In mammals, linoleic acid can be elongated and desaturated through a serious of enzymatic steps to form gamma-linolenic (18:3 n-6), dihomo-gamma-linolenic (20:3) arachidonic acid (C20:4 n-6, AA) and adrenic acid (22:4 n-6) (Figure 2.2). Linoleic acid is the precursor of arachidonic acid, a substrate for eicosanoid production, which is important for platelet and vessel wall physiology. Smaller amounts of polyunsaturated fatty acids with double bonds in the n-3 position are also present in the diet. These are referred to as (n-3) fatty acids otherwise known as omega-3 fatty acids.

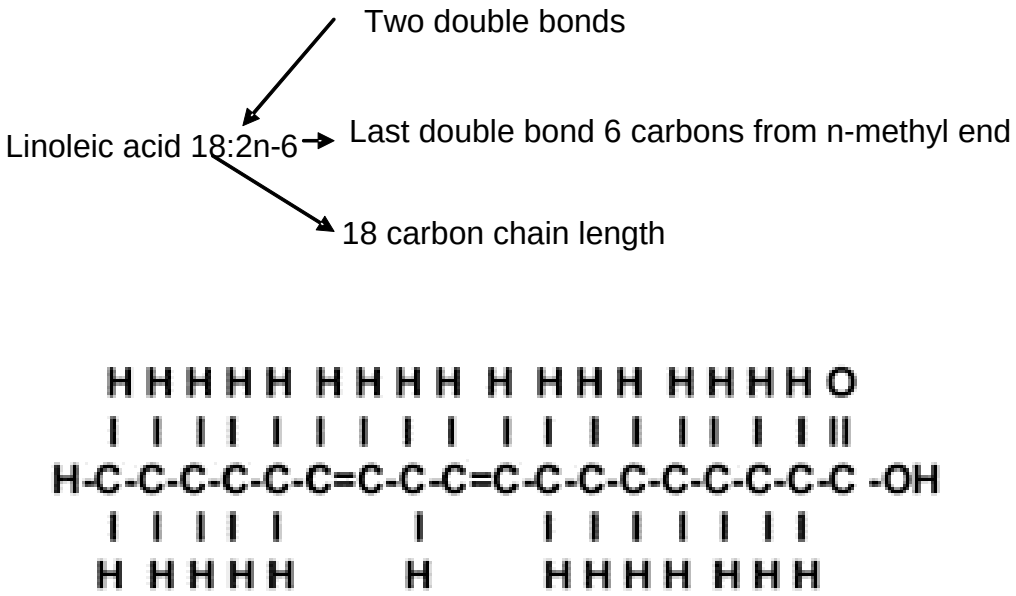


Figure 2.1 Linoleic acid (C18:2n-6). There are 18 carbon atoms in the molecule, two double bonds (=), and the last double bond is located six carbon atoms down from the omega or n-methyl end of the fatty acid.

The majority of fatty acids in tissue derive from dietary intake. However, both liver and adipose tissue are capable of de novo lipogenesis primarily from carbohydrate

sources (Wajchenberg, B.L. 2000). The majority of human studies *in vivo* suggest that diet modulates hepatic rates of *de novo* lipogenesis. Both overfeeding, particularly with excess energy derived from carbohydrates, and eucaloric low fat and high carbohydrate diet were shown to increase hepatic *de novo* lipogenesis (Diraison, F. *et al.* 2003; Hellerstein, M.K. *et al.* 1996; Hudgins, L.C. *et al.* 1996; McDevitt, R.M. *et al.* 2001). Fatty acid synthesis is accomplished from small molecule intermediates, derived from carbohydrates, amino acids and other fatty acids.

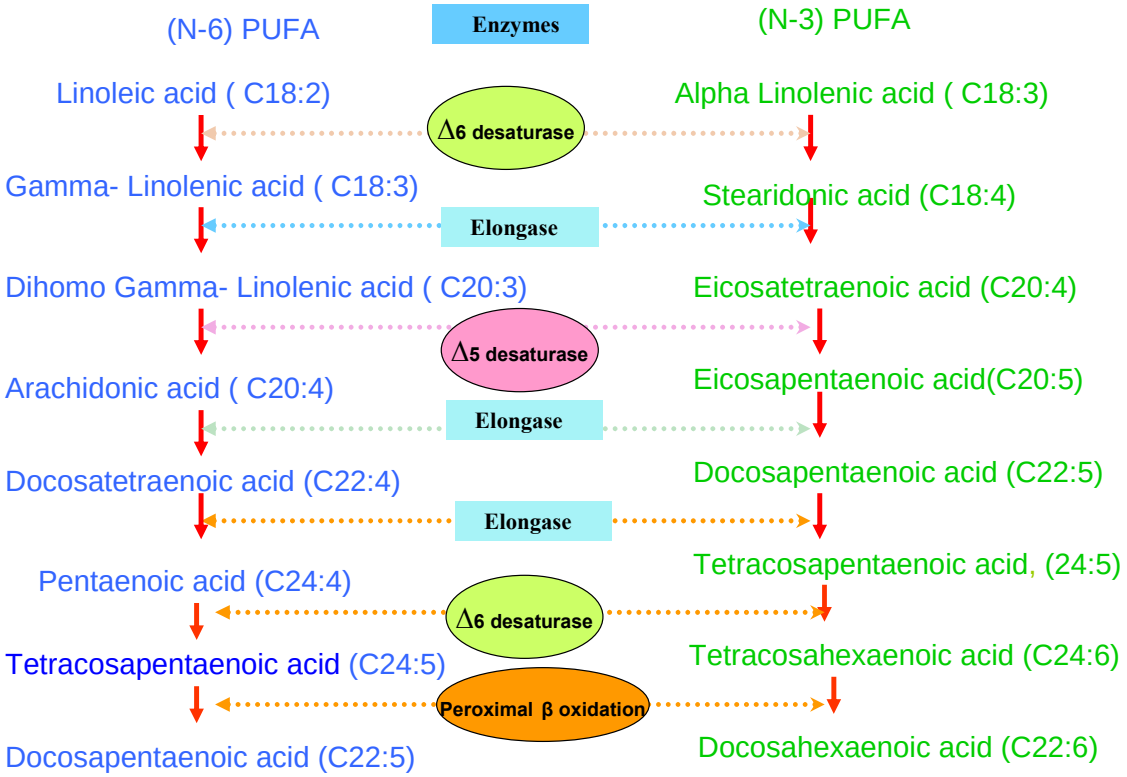


Figure 2.2 Biosynthesis of (n-6) PUFA and (n-3) PUFA from their precursors linoleic acid and alpha-linoleic acid. Steps 1-6 occur in the endoplasmic reticulum. Step 7 occurs in peroxisomes.

First, the saturated straight chain C16 palmitic acid is synthesized from acetyl CoA, which is the source of all C atoms for this synthesis. The fatty acids are made by

sequential addition of 2-C units to the “activated-COOH” end of the growing chain. The enzymes needed are “acetyl CoA carboxylase” and “fatty acid synthase”. More enzymes are produced on a high carbohydrate or fat-free diet. All other *de novo* produced fatty acids are made by modification of palmitic acid by elongation, desaturation, and hydroxylation. Humans do not have enzymes to introduce double bonds beyond carbon 9 (counting from COOH group) in fatty acid chains. Therefore, the highly unsaturated fatty acids, such as (n-6) PUFA and (n-3) PUFA, cannot be synthesized denovo from precursors in the body, and are thus considered the essential fatty acids; essential in the sense that they must be provided in the diet. Furthermore, the (n-6) PUFA and (n-3) PUFA cannot be interconverted in humans (Budowski, P. & Crawford, M.A. 1985).

The three main (n-3) PUFA are alpha linolenic acid (ALA), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). The first of the (n-3) fatty acids is alpha linolenic acid (C18:3 n-3), the parent molecule of this family of fats. Vegetable oils are the predominant sources of alpha linolenic acid. Alpha linolenic acid is found in legumes, seeds of flax, walnuts, pinto bean, soy bean, canola, and in small amounts in green leafy vegetables such as leeks, spinach, parslane (Simopoulos, A.P. 1989). They are often available as pure oils or incorporated into margarines. Alpha linolenic acid is an important plant–based source of (n-3) PUFA for vegetarians and nonseafood consumers. Animals lengthen and add double bonds (see Figure 2.2) to this eighteen-carbon fatty acid to make an entire family of (n-3) fatty acids. Of this family, eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are the most biologically active in the tissues, however, conversion of alpha linolenic acid to EPA in humans

is limited and further transformation to DHA is very low. Women, generally have better fractional conversion of alpha linoleic acid to longer chain (n-3) PUFA than in men (Pawlosky, R.J. *et al.* 2001). This may be due to a regulatory effect of estrogen. During pregnancy or lactation, alpha linolenic acid conversion is upregulated. However, the synthesis from alpha linolenic acid to longer chain (n-3) PUFA is still insufficient to meet the demand. The degree of conversion of alpha linoleic acid to EPA and DHA also varies according to the intakes of other fatty acids (Pawlosky, R.J. *et al.* 2001). It is believed that there is a competition between (n-3) and (n-6) fatty acids for the desaturation enzymes (see Figure 2.2).

There is also further evidence that delta six desaturase decreases with age (de Gomez Dumm, I.N. & Brenner, R.R. 1975). Overall, alpha linolenic acid is a limited source of (n-3) PUFA in humans. Therefore, adequate intakes of (n-3) PUFA are important for maintaining optimal tissue function. The EPA and DHA are found predominantly in oily fish, such as mackerel, herrings, sardines, salmon and tuna and other sea food (Simopoulos, A.P. 1991). The DHA is the final product of the (n-3) class (see Figure 2.2 for its structure) and the longest, most unsaturated fatty acid (see Figure 2.3) normally encountered in our diets (Sprecher, H. 2000). The DHA is found in its highest concentration in the membranes of the cells of the brain (O'Brien, J.S. & Sampson, E.L. 1965), retina (Anderson, R.E. *et al.* 1970), testes and sperm (Poulos, A. *et al.* 1973) in mammals including humans, but also reaches high level in the heart.

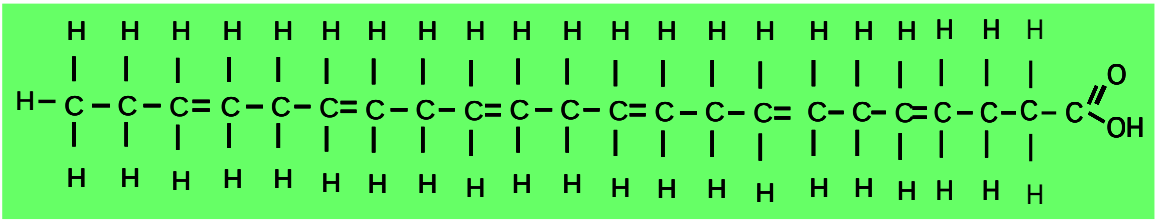


Figure 2.3 Docosahexaenoic acid (C22:6n-3; DHA)

2.1.2 Effects of habitual (n-3) PUFA in the human diet

A growing body of evidence from epidemiological studies indicates an inverse relationship between dietary fish or fish oil intake and mortality from heart disease (He, K. *et al.* 2004; Mozaffarian, D. *et al.* 2005c). Fish consumption is associated with a reduced risk from all-cause, ischemic heart disease and stroke mortality across 36 countries (Zhang, J. *et al.* 1999).

A correlation between (n-3) PUFA fatty acids in the diet and low mortality rate from coronary heart disease was first recognized in Greenland Eskimos in the 1970s (Bang, H.O. *et al.* 1976). The high level of (n-3) PUFA from marine sources in the diet of the Eskimos compared to that of Danes was inversely related to rates of myocardial infarction. Since these discoveries in the 1970s, numerous epidemiological studies have determined the relative effects of fish oil on cardiovascular disease morbidity and mortality across different populations of the world. They have further established that Greenland Eskimo (Bjerregaard, P. *et al.* 1997), Japanese (Iso, H. *et al.* 2006) and Greek (Panagiotakos, D.B. *et al.* 2005) populations have a low mortality rate from coronary heart disease, in spite of a high

intake of total fat in their diet. This has been attributed to their consumption of fish or fish-eating mammals, resulting in high consumption of long-chain (n-3) PUFA, which contains EPA, and DHA. Kromhout and co-workers (Kromhout, D. *et al.* 1985) showed a decrease in coronary heart disease mortality in people consuming relatively small amounts of fish (0.5g n-3 PUFA) over a 19 year period of time. This suggests that small doses over long periods may have beneficial affects.

Fish consumption is inversely related to sudden cardiac death. Several large cohort studies have demonstrated inverse associations between fish consumption and cardiovascular disease. The US Physicians' Health Study was a prospective cohort study of over 20000 US male physicians who were free of myocardial infarction, cerebrovascular disease, and cancer at baseline. During follow-up for 11 years weekly fish consumption was associated with a 52% lower risk of sudden cardiac death and 30% reduction in total mortality, compared with consumption of fish less than once per month (Albert, C.M. *et al.* 1998). Another report from the same study showed an inverse relationship between blood levels of (n-3) PUFA and risk of sudden death in these men without a prior history of cardiovascular disease (Albert, C.M. *et al.* 2002). The relative risk of sudden death among men with levels of long chain (n-3) PUFA in the highest quartile compared with the lowest quartile was a very low 0.1 after adjustment for monounsaturated and trans unsaturated fatty acids. In addition, in a 30 year follow-up of the Chicago Western Electric Study (Daviglus, M.L. *et al.* 1997), men who consumed 35g or more of fish daily, compared with those who had none, had a relative risk of death from coronary heart disease of 0.62 and relative risk of nonsudden death from myocardial infarction of

0.33. Chronic intake of fish or fish oil also has been shown to have an inverse relationship to myocardial infarct size and the incidence of large infarcts as evidenced by the frequency of Q-wave infarcts and by peak creatine kinase and lactate dehydrogenase activities after myocardial infarction (Landmark, K. *et al.* 1998a).

When patients survive a myocardial infarction, the resultant loss of functioning cardiac muscle will often degenerate into heart failure. Heart failure is a clinical syndrome that occurs in patients who, because of an inherited or acquired abnormality of cardiac structure and/or function, develop a constellation of clinical symptoms (dyspnea and fatigue) and signs (edema and rales) that lead to frequent hospitalizations, a poor quality of life, and a shortened life expectancy (Elliott M. Antman *et al.* 2008). It is estimated that there are nearly 23 million people with heart failure worldwide and that the burden of congestive heart failure will increase over the next two decades in developed countries (Levy, D. *et al.* 2002).

In order to investigate the relation between fish consumption and incidence of congestive heart failure, the usual dietary intakes of about 5000 adults were assessed using dietary questionnaires. During 12 years' follow-up, it was found that fish consumption was inversely associated with congestive heart failure. There was a 20% lower risk with intake of fish one to two times/week, 31% lower risk with intake 3 to 4 times/week and 32% lower risk with intake ≥5 times/week, compared with intake <1 time/month. Within this study, the dietary long-chain (n-3) PUFA intake was inversely associated with congestive heart failure with 37% lower risk in the highest quintile of intake compared with the lowest. It was concluded that

among older adults, consumption of tuna or other broiled or baked fish containing (n-3) PUFA, is associated with lower incidence of congestive heart failure (Mozaffarian, D. *et al.* 2005a). An intervention trial conducted during the course of this thesis research has shown that treatment with (n-3) PUFA can provide a small beneficial advantage in terms of mortality and admission to hospital for cardiovascular reasons in patients with heart failure in a context of usual care (Tavazzi, L. *et al.* 2008).

Atrial Fibrillation is an abnormal heart rhythm originating in the atria and the most common sustained arrhythmia observed clinically. It is marked by disorganized, rapid, and irregular atrial activation (Elliott M. Antman *et al.* 2008). The incidence and prevalence of atrial fibrillation rises with age, so that it affects nearly 10% of individuals over age 80 years. Atrial fibrillation itself is rarely life-threatening; however, it can have serious consequences through increased risk of thrombosis or if the ventricular rate is sufficiently rapid to precipitate hypotension, myocardial ischemia, or tachycardia-induced myocardial dysfunction (Bashore, T.M. *et al.* 2009).

Mozaffarian and co-workers also investigated the relationship of relative fish intake and incident rate of atrial fibrillation (Mozaffarian, D. *et al.* 2004). The cardiovascular health study, a population based prospective cohort study, was used to identify subjects with new-onset atrial fibrillation on the basis of hospital discharge records and annual electro-cardiograms. The fish consumption and usual dietary intake was assessed. After a follow up of 12 years, atrial fibrillation was detected in 980 subjects. It was concluded that, consumption of fish was inversely

associated with incidence of atrial fibrillation, with a 28% lower risk with intake one to four times per week, and 31% lower risk with intake more than five times per week, compared with less than one time per month.

In summary, epidemiological studies indicate that chronic intake of fish or fish oil consumption (even at a relative small dose) reduces mortality rate from all-causes, including ischemic heart disease, heart failure, atrial fibrillation and stroke. It also reduces sudden death and myocardial infarct size.

2.1.3 Effects of dietary intervention and supplementation with (n-3) PUFA

In addition to studies of habitual diet, several intervention studies have found that fish or fish oil has positive effects on people who have previously suffered from a myocardial infarction. These studies have focused on the role of fish or fish oil in secondary prevention of heart disease (Burr, M.L. *et al.* 1989b; Daviglus, M.L. *et al.* 1997; Hu, F.B. *et al.* 2002; Mizushima, S. *et al.* 1997; Valagussa, F. *et al.* 1999). One of the first trials with clinical end points was the DART (Diet and Re infarction Trial) (Burr, M.L. *et al.* 1989b) study, which involved over 2000 Welshmen with recent history of myocardial infarction. At the end of two years, there was a 29% reduction in total mortality among those advised to eat fish. The greatest benefits were seen as a reduction in fatal myocardial infarction. This observation led to the hypothesis that (n-3) fatty acids might protect the myocardium against the adverse effects of acute ischemic stress. From these results together with contemporary animal studies (McLennan, P.L. *et al.* 1988) the reduced mortality was attributed to the anti-arrhythmic affect of fish oil, rather

than its anti-thrombotic or anti-atherosclerotic properties. The largest and most well controlled intervention study was the GISSI Prevenzione trial, which for the first time specifically tested the hypothesis that (n-3) PUFA could reduce the risk for sudden arrhythmic death from coronary heart disease in high-risk patients. Over 11,000 post myocardial infarction patients received treatments. After following for 3.5 years, it was found that there was a 20% reduction of death from any cause and 45% reduction of sudden cardiac death in the (n-3) PUFA supplement group (Valagussa, F. *et al.* 1999).

Another randomized, single-blind, secondary prevention trial (The Lyon Diet Heart study) aimed at testing whether a Mediterranean–type diet reduced recurrence after a first myocardial infarction, compared with a healthy western type of diet similar to the National Cholesterol Education Program Step 1 Diet (NCEP 1993) (de Lorgeril, M. *et al.* 1999). Interest in the concept arose from a landmark dietary study, the Seven Country Study, in which a cohort from Crete had a lower mortality rate from Coronary Heart Disease compared with similar cohorts in other countries (de Lorgeril, M. *et al.* 1994). The Cretan Mediterranean diet is high in fruits and vegetables, rich in monounsaturated fatty acids, such as olive oil, and high in alpha linolenic acid. The sources of alpha linolenic acid in the Cretan diet are leafy vegetables in addition to nuts and legumes. In the Lyon Diet Heart Study, the plant derived alpha linolenic acid was supplemented in canola oil margarine, along with a Mediterranean diet pattern, including fish. After initial myocardial infarction, over 600 patients were randomly assigned for different treatments. At the end of 27 months, there was a 76% relative risk reduction in the major primary end points of

cardiovascular death and myocardial infarction in participants who followed the Mediterranean –style diet. Therefore, most myocardial infarction patients who had (n-3) PUFA whether obtained from specific advice to eat fish, general dietary advice or fish supplement had reduced risk of sudden cardiac death.

Clinical trials also demonstrated that (n-3) PUFA reduce sudden cardiac death. A systematic search was conducted on randomized controlled trials published up to June 2003, comparing any lipid-lowering intervention with placebo or usual diet with respect to mortality. In order to assess efficacy and safety of different lipid-lowering interventions based on mortality data, over 137,000 patients receiving treatment for lipid disorders were compared to controls in a total of 97 studies. Among them, only two interventions were found to be associated with significant reductions in total mortality: statins and (n-3) PUFA. The final outcome of the data analysis suggested that for most individuals, increasing the intake of long-chain (n-3) PUFA is a safe and inexpensive way to significantly reduce risk for coronary heart disease, especially sudden cardiac death (Studer, M. *et al.* 2005).

Slow heart rate is associated with low cardiovascular mortality (Kannel, W.B. *et al.* 1987). The normal human heart beats about 70 times a minute at rest and each beat originates in the SA node. The rate is slowed (bradycardia) during sleep and accelerated (tachycardia) by emotion, exercise, fever, and many other stimuli (Bashore, T.M. *et al.* 2009). In a meta-analysis of heart rate as a secondary outcome of randomized, double-blind, placebo-controlled clinical trials, fish oil consumption reduced heart rate (HR) in humans by 1.6-bpm (Mozaffarian, D. *et al.* 2005b). Although the overall effect was modest on a population level, even modest

differences in risk factors can have a significant impact on health. On the basis of work by Jouven and co-workers (Jouven, X. *et al.* 2001), this finding of a 1.6-bpm heart rate reduction with fish oil consumption would correspond to a ≈5% lower risk of sudden death. In the meta-analysis, the lowest EPA+DHA doses of ≈1 g/d apparently provided cardioprotection by lowering heart rate (Mozaffarian, D. *et al.* 2005b). This is consistent with observational studies and randomized trials indicating clinical benefits of fatty fish or fish oil consumption at relatively modest intake, approximately one to two servings per week or 500 to 1000 mg/d EPA+DHA, respectively (Burr, M.L. *et al.* 1989a; Hu, F.B. *et al.* 2002; Mozaffarian, D. *et al.* 2003; Mozaffarian, D. *et al.* 2004; Mozaffarian, D. *et al.* 2005d; Valagussa, F. *et al.* 1999). Heart rate lowering has also been observed as a primary outcome of an intervention trial using fish oil (Geelen, A. *et al.* 2005)

2.1.4 Effects of (n-3) PUFA underpinning cardioprotection

Antiarrhythmic Effects

Gudbjarnasan and Hallgrimsson suggested that fish oils might be antiarrhythmic, as prefeeding reduced mortality when rats were challenged with high doses of catecholamines (Gudbjarnason, S. & Hallgrimsson, J. 1976). Later, McLennan and his co-workers directly measured arrhythmias to study the possible antiarrhythmic effect of polyunsaturated fatty acids, including fish oils in ischemia (McLennan, P.L. *et al.* 1988). Coronary artery occlusion and reperfusion in the anesthetized rat was used as a whole animal model of arrhythmia and sudden cardiac death. Feeding rats a diet supplemented with tuna fish oil significantly reduced the incidence and

severity of arrhythmias, preventing ventricular fibrillation during both coronary artery occlusion and reperfusion, whereas sunflower oil, rich in (n-6) PUFA, was less effective and only reduced the incidence of ventricular fibrillation in occlusion, not in reperfusion. They concluded that dietary fat can modify the vulnerability of the myocardium to arrhythmic stimuli. The efficacy of tuna fish oil in reducing vulnerability to both ischemic and reperfusion arrhythmias suggested a potential beneficial effect of dietary (n-3) PUFA that was different and in addition to their influence on hemostasis, plasma lipids, and atherosclerosis and that may contribute to their proposed role in lowering cardiovascular disease mortality and morbidity (McLennan, P.L. *et al.* 1988).

Subsequently, McLennan reported that, more than 40% of the animals subjected to coronary occlusion, died of sustained ventricular fibrillation when fed a diet supplemented with saturated fat providing 12% of energy calories. This was not significantly reduced by an olive oil diet, whereas a group receiving a tuna fish oil diet had no arrhythmic death (McLennan, P.L. 1993). This study established that (n-3) PUFA are antiarrhythmic whereas monounsaturated olive oil are not. Similar findings have also been reported using nonhuman primates (McLennan, P.L. *et al.* 1992a) and in other rat studies (Anderson, K.E. *et al.* 1996; Hock, C.E. *et al.* 1990; Kinoshita, I. *et al.* 1994; McLennan, P.L. *et al.* 1985; Yang, B. *et al.* 1993).

A systematic review of the literature on controlled animal studies assessed the effects of (n-3) fatty acids on a range of arrhythmia outcomes (Matthan, N.R. *et al.* 2005). Summarizing the results from 13 feeding studies that compared fish oils,

EPA, and/or DHA to (n-6) PUFA, monounsaturated fatty acids, saturated fatty acids, or no treatment controls across various animal species, they concluded that fish oil supplementation is antiarrhythmic. This antiarrhythmic effect of fish oil is depicted by its ability to reduce ventricular tachycardia and ventricular fibrillation in ischemia-induced arrhythmia models. In contrast, feeding diets rich in the short chain (n-3) PUFA, alpha linoleic acid, had no effects on arrhythmia (Matthan, N.R. *et al.* 2005). The above studies fed fish oil for periods of time ranging from 4-129 weeks and relied on incorporation of (n-3) PUFA, DHA into the heart muscle membrane (Pepe & McLennan, P.L. 1996).

The antiarrhythmic action of (n-3) PUFA was also studied directly using cultured neonatal cardiomyocytes (Kang *et al.* 1994; Kang *et al.* 1995b; Kang *et al.* 1995a; Kang *et al.* 1996; Li, Y. *et al.* 1997; Xiao, Y. *et al.* 1995). In these studies, slowing of the beating rate of the myocytes was observed when micromolar concentrations of EPA or DHA were added to the medium bathing the isolated heart cells (Kang *et al.* 1994). When delipidated bovine serum albumin (BSA) was added to the superfusate, the EPA or DHA was extracted from the heart cells, and the beating rate returned to the control rates (Kang *et al.* 1994). EPA was also found to inhibit a range of toxic agents known to produce fatal arrhythmias. These included: increased extracellular Ca^{2+}, the cardiac glycoside ouabain (Kang *et al.* 1994), isoproterenol (Kang *et al.* 1995a), lysophosphatidylcholine and acylcarnitine (Kang *et al.* 1996), thromboxane, and even the Ca^{2+} ionophore A23187 (Kang *et al.* 1996). All of these agents induced tachyarrhythmias in isolated myocytes. When EPA or DHA was added to the superfusate, the beating rate slowed, and when the high Ca^{2+} or ouabain was added in the presence of the EPA, no arrhythmia was induced.

Furthermore, after a severe rhythm disturbance was induced in the cells by either elevated calcium or ouabain, addition of EPA stopped the arrhythmias, and the cells resumed their regular contractions. The addition of the delipidated bovine serum albumin to remove the free fatty acid from the myocytes resulted in recurrence of the arrhythmia. From these experiments, it appeared the free fatty acids act directly on the heart cells and need only partition (dissolve) into the hospitable hydrophobic interior of phospholipids of the plasma membranes of myocytes to elicit their antiarrhythmic actions. Finally, Leaf and Kang concluded that the arrhythmias induced in the isolated neonatal rat cardiomyocytes could be prevented in every instance by the prior addition of EPA or DHA to the superfusate bathing the cells, and the addition of EPA or DHA after an arrhythmia was induced would stop the arrhythmia. The antiarrhythmic effect of fish oil is also demonstrated in isolated rat heart models (Pepe & McLennan, P.L. 1996) and in dogs after infusion (Billman, G.E. *et al.* 1999). In conclusion, (n-3) PUFA has antiarrhythmic properties when added directly or after incorporation into all membranes. However, because so many cellular processes are altered with acute administration of fatty acids to isolated cells and not all can be re-produced with dietary studies, many may be non-specific and non representative of what occurs following dietary incorporation into cell membranes (McLennan, P.L. 2004). The dietary effects appear to rely upon stabilization of calcium handling (McLennan, P.L. 2004; McLennan, P.L. & Abeywardena, M.Y. 2005)

Other cardiovascular effects of (n-3) PUFA

Antithrombotic Effects

Thrombosis is the formation of a blood clot at the site of tissue or vascular injury. It involves different cells (platelets, leukocytes, and endothelial cells) and a variety of specific plasma proteins coagulation factors (Furie, B. & Furie, B.C. 1992). This process forms the initial plug that stops blood loss from a damaged blood vessel and is essential to repair cuts, wounds and maintain blood vessel integrity but potentially fatal if a large clot forms in, or is delivered to blood vessel of the heart, lung, and brain. Blood clot or thrombus formation is a crucial event in coronary heart disease and its occurrence can completely block a blood vessel, stop oxygen to part or all of an organ. When it occurs in coronary vessels, the result is a heart attack. An increase in the tendency for platelets to aggregate has been associated with a high frequency of myocardial infarction and sudden cardiac death (Tofler, G.H. *et al.* 1987). The dissolution of clots already formed has been effective in reducing concurrent heart attacks and sudden death (Loeliger, E.A. *et al.* 1967).

Platelet aggregation requires fibrinogen from the circulation and is stimulated by thromboxane A_2, a powerful vasoconstrictor and platelet aggregator synthesised by the platelet itself. Platelet clumping tendencies are opposed by the metabolic products of endothelial cells, particularly prostacyclin (PGI_2), that diminishes platelet aggregation and stimulate vasodilatation (Elliott M. Antman *et al.* 2008; Vane, J.R. *et al.* 1990).

Two of the best known inhibitors of prostaglandin synthesis are aspirin and EPA (Lorenz, R.L. *et al.* 1984; Nelson, G.J. *et al.* 1991). Aspirin acts by inhibiting the activity of the cyclooxygenase enzyme, whereas EPA appears to compete with arachidonic acid for the enzyme (Kinsella, J.E. *et al.* 1990). When cyclooxygenase uses EPA as a substrate, it forms different species of thromboxane and prostaglandin, thromboxane A_3 and prostaglandin I_3 (Fischer, S. & Weber, P.C. 1984; Whitaker, M.O. *et al.* 1979). Thromboxane A_3 is only weakly pro-aggregatory, whereas prostaglandin I_3 is just as potent as prostacyclin in preventing platelet aggregation (Fischer, S. & Weber, P.C. 1984; Whitaker, M.O. *et al.* 1979). In a sense, the balance of proaggregatory and antiaggregatory prostaglandins is critical in maintaining normal homeostasis.

The antithrombotic effects of (n-3) PUFA first observed as excessive bleeding in Greenland Eskimos are due to inhibition of platelet aggregation (Agren, J.J. *et al.* 1997; Mori, T.A. *et al.* 1997), probably caused by a decrease in thromboxane A_2 and an increases in PGI_3 production. A decrease in whole blood viscosity also induces a modest prolongation of bleeding time. However, modest bleeding is only evidenced by an approximate intake of (n-3) PUFA more than 3g/d. There has been no reports of serious bleeding (Knapp, H.R. 1997). Moderate consumption of fish appears to present no risk of greatly prolonged bleeding time in healthy people and evidenced by epidemiological studies which demonstrated no reduced platelet aggregation in individuals. Some evidence indicates that (n-3) PUFA supplementation may also enhance fibrinolysis or clot breakdown in normal subjects (Barcelli, U. *et al.* 1985). Other studies also show that (n-3) PUFA has an important role in establishment of plaque stability and thus reduction in the risk

neurological events in patients with advanced carotid atherosclerosis (Thies, F. *et al.* 2003). In summary, moderate consumption of fish has antithrombotic effect without causing any prolonged bleeding.

Hypolipidemic Effects

The hypolipidemic effects of (n-3) PUFA are well established. Consistent observation from many studies is that (n-3) PUFA lower plasma triglyceride levels in both healthy and hyperlipidemic subjects (Harris, W.S. 1989). The (n-3) PUFA consistently lowers serum triacylglycerol concentrations in hypertriglyceridemic patients (Harris, W.S. 1997), whereas the (n-6) PUFA do not and may even increase them (Phillipson, B.E. *et al.* 1985). Simons and co-workers (Simons *et al.* 1985) reported a decrease of 58% of plasma triglyceride in patients with type-V hyperlipidemia, and Phillipson and co-workers (Phillipson, B.E. *et al.* 1985) observed a drop of 79% in similar patients. A dose response relationship exists between plasma lipid lowering and (n-3) PUFA intake (Harris, W.S. 1997) and habitual (n-3) PUFA consumption decreases postprandial triacylglycerol concentration (Harris, W.S. *et al.* 1988). The higher doses (3 to 4 g/d) provided as supplements can reduce plasma triglyceride levels in patients with hypertriglyceridemia (Harris, W.S. 1997). In addition, Nestel reported that consumption of high amounts of fish oil prevented the expected rise in plasma cholesterol concentrations in humans due to saturated fatty acid replacement of polyunsaturated fatty acids (Nestel, P.J. 1986). Human studies have shown that fish oil reduces the rate of hepatic secretion of triacylgcerol and very low density lipoprotein in normolipidemic subjects (Harris, W.S. & Muzio, F. 1993). The

lowest lipid and lipoprotein levels were achieved with a diet low in saturated fatty acids and high in (n-3) PUFA (Nordoy, A. *et al.* 1993). While some reported that both EPA and DHA have serum triglyceride-lowering properties (Grimsgaard, S. *et al.* 1997), others claimed that only EPA lower triglycerides levels, whereas DHA appears to have little or no effect (Bonaa, K.H. *et al.* 1992a, 1992b; Willumsen, N. *et al.* 1993). In humans, the effects of consuming fish or fish oil on total cholesterol and low density lipoprotein cholesterol are minimal (Harris, W.S. 1989). However, it has been reported in non-human primates that dietary fish oil reduce the size of LDL particle and reduce its transition temperature (Parks, J.S. & Bullock, B.C. 1987). If cholesterol esters from fish oil-derived LDL deposited in the atherosclerotic plaques, this may result in a potential for cholesterol efflux from arterial plaques (Glick, J.M. *et al.* 1983). In summary, (n-3) PUFA has dose-dependent lipid lowering effects.

Antihypertensive Effects

Differences in blood pressure among populations have been known for decades, but causes of the variations are less certain. In Western countries, blood pressure rises with age, but in populations where habitual salt intake is less than 4500mg per day, average blood pressure is low, hypertension rare, and blood pressure does not rise with age (Page, L.B. 1979). There appears to be no single cause of hypertension but several body systems are in collusion: renal, cardiovascular, endocrine, and nervous. Interaction among many outside factors, including obesity, consumption of sodium, potassium and calcium, dietary fat, and perhaps certain fatty acids, alcohol intake as well as stress, make individuals susceptible. The major risk from high blood pressure is stroke. Worldwide prevalence estimates for hypertension

may be as much as 1 billion individuals, and approximately 7.1 million deaths per year may be attributable to hypertension (WHO 2002).

Evidence from laboratory investigation, observational studies, and clinical trials indicates that (n-3) PUFA can contribute to prevent the development of hypertension. The extent of antihypertensive effect seems to be dependent on the doze of (n-3) PUFA and the degree of hypertension (Appel, L.J. *et al.* 1993; Howe, P.R. 1997; Rousseau, D. *et al.* 2001). For example blood pressure was decreased about 5mmHg in trials of untreated hypertensives given >3g/d of omega-3 fatty acids (Appel, L.J. *et al.* 1993). Likewise, in a meta analysis, Morris and co-workers reviewed over 30 placebo-controlled trials on (n-3) PUFA and blood pressure in both healthy subjects and patients (Morris, M.C. *et al.* 1993). They found a statistically significant, dose-dependent reduction in blood pressure that averaged-3.0/1.5mmHg (systolic/diastolic) with an average daily dose of 4.8g of (n-3) PUFA per day. When the analysis examined the studies grouped according to type of subject, they found that the strongest effect of fish oil occurred among patients with hypertension. The effect on healthy people was not statistically significant. Among hypercholesterolemic patients, the combined analysis of six trials provided a significant effect of fish oil on reducing systolic blood pressure. Among cardiovascular patients, fish oil reduced blood pressure but the overall effect did not yield statistical significance. Morris and co-workers further observed that fish oil had no effect on blood pressure in studies using doses of (n-3) PUFA less than 3g/day (Morris, M.C. *et al.* 1993). Among EPA and DHA, one study suggested that DHA seems to be more effective in lowering blood pressure than EPA (Mori, T.A.

et al. 2000) and the other animal study suggested that only DHA has blood pressure lowering effect when the cardiac adrenergic system is the dominant mechanism involved in hypertension. However, when hypertension largely involves the renin-angiotensin system, EPA is also effective (Rousseau-Ralliard, D. *et al.* 2009).

In summary, (n-3) PUFA has antiarrhythmic, bradycardic, antithrombotic, hypolipidemic and antihypertensive effects. However, only the antiarrhythmic and bradycardic effects are demonstrable at the low intake associated with reduced cardiovascular mortality observed in epidemiological studies. These key effects are both dependent on changes in fatty acid composition and altered physiological properties of the heart, rather than vascular or circulatory effects such as atherosclerosis.

2.2 ISCHEMIC PRECONDITIONING

2.2.1 Ischemic preconditioning and its cardioprotective effects

Ischemic preconditioning was discovered and named in 1986 by Murry and co-workers (Murry, C.E. *et al.* 1986). Murry *et al* reported that anesthetized dogs subjected to 40 minutes of circumflex artery occlusion and reperfusion developed a markedly smaller myocardial infarct (reduced by 75%), if the dogs first received several brief episodes of myocardial ischemia and reperfusion just before the 40 minutes sustained occlusion.

Subsequently, other cardioprotective effects of ischemic preconditioning were established, particularly prevention of cardiac arrhythmias. Shiki and Hearse (Shiki, K. & Hearse, D.J. 1987) first demonstrated that preconditioning dramatically limits reperfusion-induced arrhythmias after short periods of ischemia in the rat heart. Hagar and co-workers (Hagar, J.M. *et al.* 1991) confirmed this observation and showed that preconditioning limited ischemia as well as reperfusion-induced arrhythmias. Other reports of arrhythmia prevention followed (Li, Y.W. *et al.* 1992; Vegh, A. *et al.* 1994)

Since the discovery of this protective phenomenon, ischemic preconditioning has been reproduced in different laboratories (Kloner, R.A. *et al.* 1998; Lawson, C.S. & Downey, J.M. 1993) and across many species, including pigs (Schulz, R. *et al.* 1998), dogs (Vegh, A. *et al.* 1990; Vegh, A. *et al.* 1994), sheep (Burns, P.G. *et al.* 1995), rabbits (Goto, M. *et al.* 1995; Imagawa, J. *et al.* 1998), rat (Liu, Y. & Downey, J.M. 1992), and mouse (Guo, Y. *et al.* 1998). The effects of preconditioning are greatest in large-animal hearts, in which the metabolism and heart rates are lower.

Although repetitive episodes of brief ischemia are often used to induce the phenomenon, it has been found that if the duration between the last brief preconditioning ischemia and the more prolonged ischemic episode needed to induce necrosis (so called “index ischemia”) is extended from five minutes to three hours or twelve hours in the dog heart, the benefit of ischemic preconditioning disappears (Kuzuya, T. *et al.* 1993). However, if the duration between the ischemic

preconditioning episodes and the prolonged coronary artery occlusion is further extended beyond 24 hours and up to 72 hours, then the protective effect returns and infarct size is again reduced (Figure 2.3) (Baxter, G.F. *et al.* 1997; Kuzuya, T. *et al.* 1993).

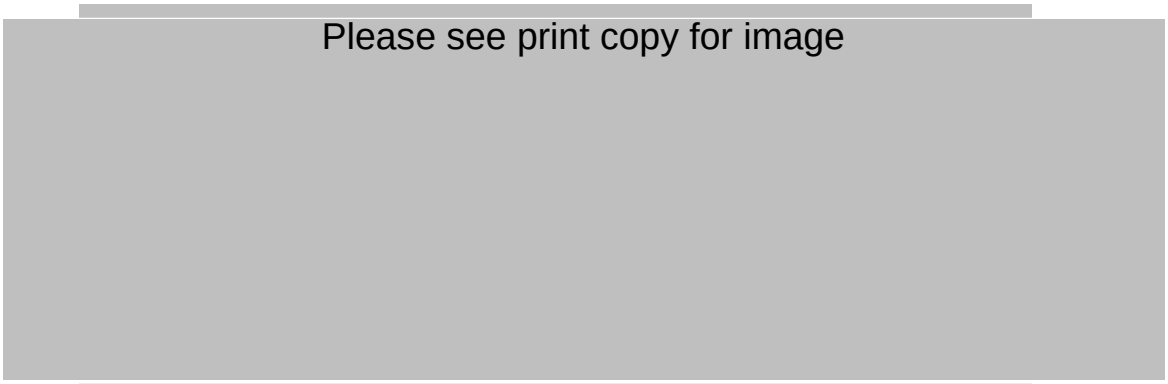


Figure 2.3 Time elapsed from preconditioning stimulus (adapted source, Yellon, D.M. & Downey, J.M. 2003)

This later phase of ischemic preconditioning is variously called, the second window of protection; delayed preconditioning or late preconditioning, whereas acute preconditioning is often referred to as classic or early preconditioning (Murry, C.E. *et al.* 1986). The biology of early preconditioning is summarized as follows: early preconditioning delays but does not prevent cell death during the index ischemia. Therefore, if the duration of the index ischemia is prolonged or reperfusion is not eventually instituted, preconditioning will not delay cell death indefinitely (Murry CE, J.R. & KA, R. 1994).

Infarct size (Yellon, D.M. *et al.* 1998) and additionally, post ischemic recovery of contractile function (Cave, A.C. & Hearse, D.J. 1992; Weselcouch, E.O. *et al.* 1995), are widely used end point of ischemic preconditioning. However, the

recovery of function after an ischemic insult is influenced by both the number of surviving cells and the degree to which they have been stunned. Myocardial stunning (see section 2.2.3) can be defined as a prolonged and fully reversible dysfunction of the ischemic heart that persists after reperfusion despite the normalization of blood flow (Thomas, M. *et al.* 2008). Unlike the infarcted heart, the stunned myocardium normally recovers fully in one to two days and stunning itself may be protective by reducing oxygen requirements.

Differences between the early and the late ischemic preconditioning

Both early and late ischemic preconditioning limit myocardial infarct size, but the early preconditioning produced greater infarct-sparing effects (Baxter, G.F. *et al.* 1997). On the other hand, late preconditioning alleviates myocardial stunning, whereas early preconditioning does not (Bolli, R. 2000). The duration of the protection obtained by the early and late phases, (two to three hours versus 24 to 72 hours) are also greatly different. Moreover, the duration of the early phase cannot be extended by continuous infusion of pharmacological triggers (pharmacological agents that precipitates other events which produce protection) (Tsuchida, A. *et al.* 1994) nor by repeating the brief ischemic preconditioning episodes (Cohen, M.V. *et al.* 1994), presumably because of desensitization of receptors. For late preconditioning, stimuli can be applied repeatedly at 48- to 72-hour intervals to maintain a continuous protective effect and are not subject to desensitisation (Baxter, G.F. *et al.* 1997; Dana, A. *et al.* 1998).

Although the signal transduction pathways leading to protection in late preconditioning share many of the steps identified for early phase protection, there are certain differences between both phases (Baxter, G.F. & Ferdinandy, P. 2001; Bolli, R. 2000). For example, unlike early protection, the late phase of protection is delayed, due to its reliance on altered expression of protective proteins (Baxter, G.F. & Ferdinandy, P. 2001).

2.2.2 The mechanism of ischemia/reperfusion injury and protection by ischemic preconditioning

With prolonged imbalance between myocardial oxygen demand and blood supply, the heart is unable to maintain its rate of cellular oxidation, leading to metabolic imbalances (Poston, J.M. & Parenteau, G.L. 1992). Sudden occlusion of a major branch of a coronary artery changes mitochondrial metabolism and function within seconds. Initially, these changes are reversible (myocardial stunning), however, if deprivation of oxygen extends to a longer period of time, these alterations progressively become more severe, leading to damage of the tissue which eventually becomes irreversible (myocardial infarction). The severity and progression of ischemia are not only determined by the extent of oxygen deprivation but also by the relative accumulation of toxic metabolites contributed by the reduction in blood flow (Hearse, D.J. 1988).

The normal function of heart muscle is supported by adequate rates of myocardial blood flow, oxygen, and combustion of fat and carbohydrates in the form of glucose and lactate. Under normal aerobic conditions, cardiac energy is derived

from fatty acids, supplying around 80% percent of the energy for adenosine triphosphate (ATP) synthesis. The rest of the energy comes from oxidation of pyruvate formed from glycolysis and lactate oxidation. Almost 98% of the ATP formed comes from oxidative phosphorylation via the respiratory chain in the mitochondria, only around two percent of ATP is synthesized by glycolysis. Approximately two-thirds of the ATP used by the heart goes to contractile shortening, and the remaining one-third is used by sarcoplasmic reticulum Ca^{2+} ATPase and other ion pumps essentially involved in relaxation (Burke, A. & Renu, V. 2008).

During ischemia, the heart adaptively undergoes metabolic changes that allow for a reduction in oxygen demand and a more efficient utilization of substrates. Myocardial cellular metabolism preferentially oxidises fatty acids for energy production. Under severe ischemic conditions, since there is no blood flow to deliver glucose to the tissue, rapid breakdown of glycogen and the increase in glucose transporters occurs, allowing for preferential use in support of ion pumps (Ferrari, R. *et al.* 1998; King, L.M. & Opie, L.H. 1998). However, as ischemia progresses, myocardium becomes more acidic due to the decreased washout of lactate and other accumulate of metabolites. This decrease in pH, accompanied by the gradual accumulation of NADH, protons, lactate and alanine inhibits ATP production via inhibition of enzymes such as pyruvate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and the citric acid cycle. This eventually depresses contractile function and changes ion homeostasis which further contributes to the deleterious sustained contracture of the cell (Ferrari, R. *et*

al. 1998; Hassinen, I.E. *et al.* 1998; Hearse, D.J. 1988; Knight, R.J. *et al.* 1996; Kuzmin, A.I. *et al.* 1998).

Without a doubt, early reperfusion is the most successful means to limit ischemia induced metabolic changes and to ensure tissue survival, whereas late reperfusion has been referred to as a "double-edge sword", in that it damages previously ischemic tissue, when in theory it should be a cell-salvaging tool (Braunwald, E. & Kloner, R.A. 1985; Ferrari, R. *et al.* 1993; Hearse, D.J. 1991). Whether cell death is entirely caused by ischemic insult or by reperfusion is still controversial and may never be answered because there can not be reperfusion without ischemia. Some studies suggested that ischemia initially sets the scene for the damage induced by reperfusion, but it alone is not sufficient enough to cause cell death (Hearse, D.J. 1991; Kloner, R.A. 1993). Ischemia-reperfusion injury in the heart is complex and mainly characterized by reversible contractile dysfunction, known as stunning, and irreversible injury leading to cardiomyocytes death and myocardial infarction (Bolli, R. 1992; Braunwald, E. & Kloner, R.A. 1982; Kloner, R.A. 1993).

Myocardial stunning and ischemic preconditioning

Myocardial stunning is defined by the mechanical dysfunction that persists after reperfusion, despite the absence of irreversible damage and despite restoration of normal coronary flow (Bolli, R. 1991). Two major hypothesis have been raised as the mechanism of myocardial stunning: the oxyradical hypotheses, in which myocardial stunning is caused by the production of reactive oxygen species; and the calcium hypothesis, in which stunning caused by a calcium overload upon reperfusion (Bolli, R. & Marban, E. 1999).

It has been proposed that formation of increased reactive oxygen species during reperfusion could directly alter contractile filaments by oxidative modification of contractile proteins. This change makes the contractile filaments less responsive to calcium. Reactive oxygen species formation also indirectly cause cellular calcium overload, with the constant contractile stimulation causing damage to the intracellular structure and contractile components of the cardiomyocytes. The slow recovery characteristic of stunned myocardium could be the result of repair of oxidative damage and/or resynthesis of contractile proteins (Bolli, R. & Marban, E. 1999).

Transient calcium overload has been implicated in the pathogenesis of stunning, and it is known that reactive oxygen species, which have been closely linked to stunning, can produce cellular calcium overload (Gao, W.D. *et al.* 1996). Although cytosolic calcium concentration rises during ischemia, neither impaired myofilament calcium responsiveness (Carrozza, J.P., Jr. *et al.* 1992; Van Eyk, J.E. *et al.* 1998) nor proteolytic degradation of the contractile protein machinery (Gao, W.D. *et al.* 1997) has been found to occur during the ischemic phase. Thus, reperfusion appears to be necessary for calcium overload to induce the mechanical abnormalities responsible for stunning. Calcium overload may activate calpains, calcium dependent neutral proteases, resulting in selective proteolysis of myofibrils (Gao, W.D. *et al.* 1997). The most interesting feature of stunning is its eventual reversibility, with a distinctive time course of recovery over several hours or days. The partially degraded contractile proteins would have to be replaced by newly synthesized ones to repair the myofilaments, and the time courses required for

resynthesis of damaging proteins would partly explain delayed recovery of function in the stunned myocardium (Bolli, R. & Marban, E. 1999).

Late ischemic preconditioning has provided significant protection against myocardial stunning. An initial episode of stunning triggers a delayed adaptive response that enables the myocardium to be more resistant to subsequent episodes of stunning 24-72 hours later (Bolli, R. *et al.* 1998).

Cell death during ischemia-reperfusion and protection afforded by ischemic preconditioning

Myocardial ischemia followed by reperfusion is known to produce cardiomyocyte death (Majno, G. & Joris, I. 1995). Oncosis, apoptosis and necrosis are the form of myocytes death during ischemia-reperfusion and occur during ischemia-reperfusion at variable degree. Apoptosis is defined as the occurrence of internucleosomal fragmentation of genomic DNA associated with a sealed plasma membrane. Oncosis is defined as the early plasma membrane rupture and disruption of cellular organelles, including mitochondria (Otani, H. 2008). Necrosis is the ultimate form of cell death and is defined as the accidental collapse of cellular homeostasis, compartmentalisation and cell membrane integrity with the release of cytosolic material and with random nuclear DNA fragmentation (Bartling, B. *et al.* 1998). The potential causes of reperfusion injury include: rapid normalization of extracellular tissue osmolarity, calcium overload, rapid normalization of extracellular pH, oxygen radical generation.

The accumulation of Na^+ and the products of anaerobic metabolism during ischemia create an intracellular and extracellular increase in osmotic load. Reperfusion normalizes the extracellular osmolality by washing out these molecules and leads to a cellular uptake of water and an increase in intracellular pressure and mechanical stress (Inserte, J. *et al.* 1997). Increase in sarcolemmal fragility combined with mechanical stressed by cell swelling during ischemia-reperfusion results in rupture of the sarcolemma and cell deterioration (Piper, H.M. & Garcia-Dorado, D. 1999).

Generally, intracellular and extracellular (free) calcium concentrations are approximately 0.1 and 1000 μmol/L, respectively. More than 99% of the total cell calcium content is bound to proteins or phospholipids, or sequestered into endoplasmic reticulum, and mitochondria. The major source of a rise in intracellular free [Ca^{2+}] is often the extracellular calcium content. However, the cell contains enough bound or sequestered calcium to increase intracellular free [Ca^{2+}]. This occurs if the bound calcium is displaced from its binding sites or if the calcium sequestered in sarcoplasmic reticulum or calcium in mitochondria is released to the cytosol (Kristian, T. & Siesjo, B.K. 1998). In normal conditions, calcium fluxes associated with signal transduction are usually well regulated by Na^+-Ca^{2+} exchange and by an ATP-driven Na^+ -$2H^+$ exchanger. However, the large increase in differences in calcium concentration (extracellular fluids having a 10000-fold higher concentration than intracellular ones) and electrical potential across plasma membranes (the inside being 60 to 90mV negative to the outside) translocate calcium into cells (Carafoli, E. 1987). The calcium transport system in the inner

membrane of mitochondria has uptake and efflux components. Uptake of calcium occurs via uniporter, driven by a large electrical potential across the inner membrane. The transfer of calcium out of the mitochondria in heart occurs mainly via the exchange of Ca^{2+} for Na^{+}, direct exchange of Ca^{2+} for H^{+} as well as the calcium dependent pore that opens at high levels of calcium (Denton, R.M. & McCormack, J.G. 1990). If the influx of calcium exceeds the capacity of extrusion pathway through Na^{+}-Ca^{2+} exchange, intramitochondrial calcium concentration increases, and calcium will be sequestered within the mitochondria. This substantial increase in calcium in mitochondria opens a large conductance pore, called mitochondrial transition pore (MPT), which allows the release of calcium as well as various ions and molecules with a molecular mass less than 1500D. In this process, mitochondria shows osmotic swelling, collapse of membrane potential, cessation of ATP production, disruption of cytosolic calcium regulation, loss of cellular energy, acidosis, thereby creating a vicious cycle (Kristian, T. & Siesjo, B.K. 1998). Increases in cytosolic calcium also activate proteases, lipases and phospholipases, which increase membrane destruction. Contractile machinery is also activated with the actin-myocin crossbridges becoming irreversibly deformed by the cell's inability to relax. Ultimately, hypercontracture of the cells leads to cellular disruption and irreversible cell death (Kristian, T. & Siesjo, B.K. 1998; Piper, H.M. & Garcia-Dorado, D. 1999). This entire process can be attributed to the simple failure to maintain calcium in its normal compartments.

After prolonged ischemia, the anaerobic metabolism and the breakdown of ATP produce an excess of H^{+}, resulting in low cytosolic pH. On reperfusion, pH is

rapidly normalized in interstitial space and generates a gradient between cytosol and interstitium. This activates a Na^+-H^+ exchanger which is required for the extrusion of H^+ and inclusion of Na^+ into the cytosol. Excess cytosolic Na^+ may activate Na^+/Ca^{2+} exchange mechanisms to remove Na^+ outward and increase the pre-existing calcium overload of the cells.

Free radicals produced during cardiac reperfusion have been implicated in cardiomyocyte death. A free radical is any organic or inorganic molecule or atom that contains an unpaired electron. Oxygen free radicals during reperfusion have been shown to disrupt sarcolemma, Na^+/Ca^{2+} exchanger and Na^+/K^+ ATPase activity. Inhibition of Na^+/K^+ ATPase activity results in Na^+ overload, which leads to activation of Na^+/Ca^{2+} exchanger and calcium overload (Piper, H.M. & Garcia-Dorado, D. 1999). It was also suggested consistently that free radicals inactivate key regulatory Krebs cycle enzyme, α-ketoglutarate dehydrogenase (Humphries, K.M. *et al.* 1998; Nulton-Persson, A.C. *et al.* 2003). This results in reduced rates of NADH-linked mitochondrial respiration and ATP synthesis (Sadek, H.A. *et al.* 2002). The depletion of cellular energy eventually causes myocyte death due to failure of ionic gradients enzyme activities and physical disruption to membranes.

The mechanism of protection of cardiomyocytes from cell death by ischemic preconditioning appears to be due to protection of mitochondria. It has been demonstrated that ischemic preconditioning reverses many aspects of mitochondrial dysfunction induced by ischemia/reperfusion including the loss in the activity of the redox sensitive Krebs cycle enzyme alpha-ketoglutarate dehydrogenase; declines in

NADH-linked ADP-dependent mitochondrial respiration; insertion of the pro-apoptotic gene Bcl-2 protein Bax into the mitochondrial membrane; and release of cytochrome c into the cytosol (Lundberg, K.C. & Szweda, L.I. 2006). Ischemic preconditioning also has been reported to enhance cell-volume regulation via activation of chloride channels and may account for majority of sarcolemma protection against reperfusion-induced cell swelling, prevention of cardiomyocytes death and myocardial infarction (Armstrong, S.C. *et al.* 2001; Diaz, R.J. *et al.* 1999; Pasantes-Morales, H. *et al.* 2006). However, the precise molecular mechanism afforded by ischemic preconditioning against myocyte death remains unclear.

2.2.3 Role of surface receptors in ischemic preconditioning

The activation of one of a few cardiomyocyte surface receptors is thought to be an initial event in ischemia that trigger the preconditioning process. Activation of G protein coupled receptors activates phospholipase C generating intracellular D-myoinositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) This activates protein kinase C, an important step for mediating ischemic preconditioning (Brooks, G. & Hearse, D.J. 1996; Simkhovich, B.Z. *et al.* 1998). Of course the preconditioning effects are overwhelmed if the ischemia is sustained for long enough to induce intracellular damage. The important role of adenosine and bradykinin receptors in triggering ischemic preconditioning have been investigated (Baxter, G.F. 2002; Cohen, M.V. *et al.* 2000; Goto, M. *et al.* 1995). Studies reported that adenosine antagonist (Auchampach, J.A. *et al.* 1997; Baxter, G.F. *et al.* 1994; Liu, G.S. *et al.* 1991; Liu, G.S. *et al.* 1994) and bradykinin antagonist

(Baxter, G.F. & Ebrahim, Z. 2002; Schulz, R. *et al.* 1998) completely abolished the infarct size reduction afforded by ischemic preconditioning.

Role of reactive oxygen species in ischemic preconditioning

The superoxide anions, hydrogen peroxide, hydroxyl radical and singlet oxygen are collectively known as reactive oxygen species. Numerous studies report that reactive oxygen species play a crucial role in the complex cascade of intracellular signal transduction mediated by ischemic preconditioning (Pain, T. *et al.* 2000; Richard, V.J. *et al.* 1988; Vanden Hoek, T.L. *et al.* 1998). It is known that reactive oxygen species, which have been closely linked to stunning, can produce cellular calcium overload (Gao, W.D. *et al.* 1996). Prior administration of antioxidants or free radical scavengers has been found to abrogate the protective effect of ischemic preconditioning (Baines, C.P. *et al.* 1997; Das, D.K. *et al.* 1999; Kevin, L.G. *et al.* 2003). For example, infusion of *N*-2-mercaptopropionyl glycine, a diffusible antioxidant (Baines, C.P. *et al.* 1997; Cleveland, J.C. *et al.* 1997), or dimethylthiourea, a radical scavenger (Das, D.K. *et al.* 1999), blocks the protection of ischemic preconditioning and allowing full expression of infarction. A unique feature of ischemic preconditioning is the "so called' cellular memory of cardioprotection, which lasts up to two to three hours after the cessation of the preconditioning stimulus (Yellon, D.M. & Dana, A. 2000). A recent report speculated that reactive oxygen species generated via the activation of mitochondrial KATP channels play an important role in this memory of cardioprotection (Stowe, D.F. & Kevin, L.G. 2004).

2.2.4 Late ischemic preconditioning and cardioprotective proteins

Late preconditioning develops 12-24 hours after the initial stimulus and lasts for 72-96 hours (Bolli, R. 2000). Because of the relative long- lasting nature of late ischemic preconditioning, numerous experiments attempted to exploit these mechanisms to protect the ischemic myocardium. Late ischemic preconditioning is thought to be triggered by nitric oxide, generated by the endothelial nitric oxide synthase. Nitric oxide acts through the generation of reactive oxygen species, and it activates a series of redox –sensitive transcription factors including NF kappa B (Dawn, B. & Bolli, R. 2002). The delay in onset is due to the requirement for new protein synthesis such as manganese superoxide dismutase (Hoshida, S. *et al.* 2002), heat shock proteins (Marber, M.S. *et al.* 1993), cyclooxygenase-2 (Shinmura, K. *et al.* 2000; Shinmura, K. *et al.* 2002), and inducible nitric oxide synthase (Dana, A. *et al.* 2001; Imagawa, J. *et al.* 1999). Studies indicated that excessive levels of reactive oxygen species generated by prolonged ischemia/reperfusion may contribute to myocardial damage, and up regulation of cellular antioxidants could represent a mechanism by which late preconditioning confers protection. Mitochondrial super oxide dismutase concentrations and activity are increased 24h following either ischemic preconditioning or administration of an adenosine A1 agonist (Yamashita, N. *et al.* 1994; Yamashita, N. *et al.* 1998). Furthermore, anti-sense inhibition of mitochondrial super oxide dismutase prevents delayed cardio protection.

Rats as well as rabbits have been used to investigate the potential of heat shock proteins to limit myocardial infarct size (Hutter, M.M. *et al.* 1994; Marber, M.S. *et*

al. 1993). Protection against myocardial infarction, as mediated by heat shock proteins enhanced synthesis following whole body heat shock or a short ischemic episode was found to be only transient (Yellon, D.M. & Latchman, D.S. 1992). A brief ischemic episode can elicit Hsp70 and Hsp60 synthesis and increase resistance against myocardial infarction, 24h later (Marber, M.S. *et al.* 1993). The improved ischemia tolerance are associated with increased Hsp70 tissue levels and a decrease in the occurrence of arrhythmias in delayed cardioprotection and ventricular fibrillation (Vegh, A. *et al.* 1994). Studies have demonstrated that ischemic preconditioning up-regulates the expression and activity of COX-2 in the heart, and that this increase in COX-2 activity mediates the protective effects of the late phase of preconditioning against both myocardial stunning and myocardial infarction (Bolli, R. *et al.* 2002). The generation of NO during the preconditioning ischemia is the trigger that initiates the development of ischemic preconditioning. It has also been demonstrated that the generation of NO during the subsequent ischemic episode 24 hours later mediates the cardio protective effects of ischemic preconditioning (Bell, R.M. *et al.* 2002).

2.2.5 Preconditioning mimetic agents mediating cardioprotection

Although advances have been made in understanding the mechanisms of ischemic preconditioning, the inability to implement ischemic preconditioning before myocardial infarction is limiting clinical application. The G-protein coupled receptor agonists, such as adenosine and bradykinin have so far been effective in exerting cardioprotection against ischemic reperfusion injury. However, the

initiation of early preconditioning with a single drug causes marginal cardio protection (Belhomme, D. *et al.* 2000).

Preconditioning mimetic actions are achieved activation of either sarcolemmal or mitochondrial K_{ATP} channels (Gross, G.J. & Fryer, R.M. 1999; Gross, G.J. 2000) For example, The diuretic, smooth muscle relaxing potassium channel activator diazoxide, SB203580, is believed to primarily open mitochondrial KATP channels (Garlid, K.D. *et al.* 1996; Liu, Y. *et al.* 1998) and its preconditioning effects are prevented by 5-hydroxydecanoate, a potent closer of mitochondrial channels (Liu, Y. *et al.* 1998). However, HMR 1098 or 1883 which close only surface channels (Gogelein, H. *et al.* 1998; Sato, T. *et al.* 2000). Prevent diazoxide-mediated protection (Gross, G.J. 2000). Furthermore, 5-hydroxydecanoate, a drug reported to close only mitochondrial K_{ATP} channels, prevented the ischemia-induced shortening of action potential duration, which is associated with opening of sarcolemmal K_{ATP} channels (Bernardo, N.L. *et al.* 1999). These findings indicate the lack of specificity of diazoxide and the drugs used to define its mechanisms of action. Inhibitors of mitogen-activated protein kinases (MAP kinase) are also cardioprotective but lack specificity for individual isoforms. The p38 MAP Kinase at least has five isoforms, although only p38 alpha and beta are expressed within the heart (Saurin, A.T. *et al.* 2000). In rat cardiomyocytes, p38 alpha mediates apoptosis whereas p38 beta is anti-apoptotic (Wang, Y. *et al.* 1998). In isolated rat hearts, blockade of p38 alpha and beta with SB203580 does not affect the infarct size reduction induced by ischemic preconditioning (Schneider, S. *et al.* 2001).

However, SB203580 blocks protection of ischemic preconditioning in rabbit cardiomyocytes (Armstrong, S.C. *et al.* 1999).

In addition, the potential side effects of ischemic preconditioning agents are not negligible. The adenosine receptor agonists as preconditioning mimetic agents produce substantial hemodynamic effects including bradycardia and hypotension, which limit their widespread clinical use (Shryock, J.C. *et al.* 1998). The opioid agonists used in clinical medicine including morphine, levorphanol, meperidine, fentanyl, and methadone can activate cell surface receptors to produce cardioprotection. They are widely used for the treatment of pain. However, the US Federal Drug Administration has not approved these drugs for use in patients with unstable angina or who have existing myocardial infarction. This is likely due to the high potential for dependence and toxicity of this drug (Fryer, R.M. *et al.* 2002). Regular administration of nitroglycerin has also been used as a therapeutic means which may work to reproduce late cardioprotection. However, continuous administration of nitric oxide releasing agents results in the rapid development of tolerance to their vasodilator and anti-ischemic effects (Hill, M. *et al.* 2001).

The schedule of administration is crucial for a cardioprotective drug. The preconditioning treatment must be administered before the onset of ischemia. Unfortunately, it is rarely possible to predict an impending thrombus in patients leading to acute myocardial infarction. Even when prior treatment with the pharmacological preconditioning agent is feasible, the duration of the protection afforded is limited. According to experimental evidence in laboratory animals, the

protective effects of preconditioning is unlikely to exceed 48 to 96 hours (Bolli, R. 2000).

In summary, despite advances in identifying pharmacological approaches to mimic ischemic preconditioning, their lack of cardiac specificity and inherent potential for side effects and poor potential to predict ischemic episodes limits the application of such approach. Neither brief episodes of ischemia nor pharmacologic agents that mimic ischemia are routinely used as therapy in patients in the clinical setting (Kloner, R.A. 2006; Kloner, R.A. & Rezkalla, S.H. 2006).

2.3 PARALLEL CARDIOPROTECTIVE EFFECTS OF (N-3) PUFA MEMBRANE ENRICHMENT AND ISCHEMIC PRECONDITIONING

Fish oil prevents fatal arrhythmias (Billman, G.E. *et al.* 1999; Kang *et al.* 1994, 1995a; Matthan, N.R. *et al.* 2005; McLennan, P.L. *et al.* 1988; McLennan, P.L. *et al.* 1996) and decreases cardiac oxygen consumption (Pepe, S. & McLennan, P.L. 2002). Parallel to this, ischemic preconditioning also reduces arrhythmias (Hagar, J.M. *et al.* 1991; Li, Y.W. *et al.* 1992; Shiki, K. & Hearse, D.J. 1987; Vegh, A. *et al.* 1992), improves heart function and is related to post-ischemic myocardial stunning (Bolli, R. *et al.* 1989), which is associated with lower oxygen demand. In spite of having more susceptibility to peroxidation than other fatty acids (Luostarinen, R. *et al.* 1997) (n-3) PUFA are cardio protective. This may be due to the adaptive response of an increase in antioxidants caused by an increase in

oxidative free radical production (reminiscent of those achieved by ischemic preconditioning).

2.4 THESIS HYPOTHESIS AND AIMS

Based on the summary information above, this study hypothesized that the long chain (n-3) PUFA provide preventative cardioprotection (reduced infarct, arrhythmia prevention, improved cardiac function). This is achieved through incorporation into cardiac membrane and up regulation of endogenous antioxidant systems in the myocardium and reduced oxygen demand. In addition, (n-3) PUFA enriched hearts may share some common mechanisms of action with ischemic preconditioning on the levels of protein expression. This study aims to increase understanding of the cardioprotective mechanisms of long chain omega-3 fatty acids to establish a rationale for their use in primary prevention of cardiovascular disease morbidity and mortality.

Specifically it:

Determines the relationship between cardiac membrane fatty acid composition and the cardio protection attributable to diet and preconditioning (Chapter Four).

Evaluates the effects of diet and preconditioning and their interactions on heart function (Chapter Five).

Measures oxidation products and antioxidants formed in the heart during oxidative stress caused by ischemia/ reperfusion and their modulation by diet and ischemic preconditioning (Chapter Six).

CHAPTER 3

GENERAL METHODS

3.1 INTRODUCTION

The heart must pump continuously throughout life. Components of successful pumping depend on regular rhythm, contractile properties of the heart muscle and continuous supply of oxygen and nutrients via coronary arteries. Acute coronary occlusion inhibits myocardial contractility. If left untreated for a sufficient period, myocardial infarction occurs. In myocardial infarction, the fundamental alteration is loss of functioning myocardium. With increasing size of the infarcted myocardium, left ventricular pump function decreases. Left ventricular end-diastolic pressure and left ventricular end-systolic volume increase. Cardiac output, stroke volume, and blood pressure may decrease (Ganong, W.F. 2007).

The fundamental principle of modern therapy for acute myocardial infarction is based on the evidence that it is frequently associated with thrombotic coronary artery occlusion (Blumgart, H.L. *et al.* 1941; DeWood, M.A. *et al.* 1980). One approach to the treatment of occlusive thrombosis consists of pharmacological dissolution of the blood clot by intravenous infusion of plasminogen activators that activate the fibrinolytic system (Collen, D. 1996; Moreadith, R.W. & Collen, D. 2003). Fibrinolytic agents activate plasminogen to the active enzyme plasmin,

which in turn digests fibrin to soluble degradation products. The clinical importance of reperfusion therapy is early achievement of artery patency. Without reperfusion, cell death and necrosis will occur. Early coronary reperfusion of the blocked coronary artery results in myocardial salvage. It also decreases left ventricular dysfunction, and increases the long-term prognosis for survival in patients with coronary artery disease (Braunwald, E. 1993; Brouwer, M.A. *et al.* 1996; Cannon, C.P. 1999; Simes, R.J. *et al.* 1995; Steg, P.G. *et al.* 2005; The, G.I. 1993). Reperfusion also limits the extent of irreversible myocardial injury in experimental animals (Jennings, R.B. *et al.* 1960; Jennings, R.B. *et al.* 1985).

Extensive efforts have been focused on the salvaging of jeopardized cardiomyocytes by reducing the progression of irreversible injury due to ischemia. It has been found that repeated short episodes of ischemia, which individually have not been long enough to cause tissue necrosis, can actually render the heart more resistant to ischemic insult. For example, in a study conducted by Geft *et al*, the effects of brief intermittent periods of ischemia on myocardial viability were investigated (Geft, I.L. *et al.* 1982). Brief periodic coronary occlusions of the left anterior descending coronary artery were produced up to 18 times by inflating and deflating the balloon of an intracoronary catheter in dogs for periods of 5-15 minutes, followed by 15-minute periods of reperfusion. Using creatine kinase release, and triphenyl tetrazolium chloride staining, and light and electron microscopy, they observed that many short episodes of ischemia could have a cumulative effect and cause necrosis where any single episode would not. However, they also observed that in many dogs the opposite occurred where no necrosis was evident. They concluded that intermittent reperfusion could have a

beneficial effect and may prevent necrosis, even when total occlusion time exceeded 200 minutes.

The single greatest advance in salvaging jeopardized cells was the discovery in 1986 by Murry *et al*, when they tested the hypothesis that intermittent reperfusion may be beneficial to the myocardium by washing out catabolites that have accumulated during ischemia (Murry, C.E. *et al.* 1986). To test this hypothesis, two sets of experiments were performed. In the first set, one group of dogs was pre treated with four cycles of five minutes circumflex artery occlusions, each separated by five minutes of reperfusion, followed by a sustained 40 minutes occlusion. The control group received a single, sustained, 40 minutes occlusion. In the second set, an identical pre-treatment protocol of short circumflex artery occlusions was followed before animals received a sustained three hours occlusion. Control animals received a single three hours occlusion. Animals were allowed four days of reperfusion thereafter and histologic infarct size was then measured. Dogs subjected to 40 minutes ischemia but pre-treated with the short periods of ischemia, paradoxically had infarct size limited to 25% of that seen in the control group that was subjected to the 40 minutes ischemia only. There was no protection observed with the very long three hours occlusion.

This phenomenon of protecting myocardium against irreversible damage during a period of sustained ischemia by preceding periods of brief ischemia has been termed ischemic preconditioning. For the first time, it was shown that infarct size limitation was possible. In fact, so powerful was the observed protection that this phenomenon has been recognized as "the strongest form of *in vivo* protection

against myocardial ischemic injury other than early reperfusion" (Kloner, R.A. *et al.* 1998). The preconditioning phenomenon was reproduced by a multitude of experimental laboratories (Kloner, R.A. *et al.* 1998) in different animal models (Lawson, C.S. & Downey, J.M. 1993), and subsequently was shown to occur in a variety of species including rat (Liu, Y. & Downey, J.M. 1992), rabbit (Baxter, G.F. & Yellon, D.M. 1999; Urabe, K. *et al.* 1993), pig (Schott, R.J. *et al.* 1990), and dog hearts (Li, G.C. *et al.* 1990). There is now compelling evidence that it exists in humans. This evidence arises from *in vitro* experiments with human ventricular myocytes (Ikonomidis, J.S. *et al.* 1994), human atrial trabeculae (Walker, D.M. *et al.* 1995), ventricular trabeculae (Cleveland, J.C., Jr. *et al.* 1996), and studies of cultured myocytes from patients undergoing planned procedures, which invariably involve brief periods of ischemia, such as percutaneous transluminal coronary angioplasty (Deutsch, E. *et al.* 1990). This acute adaptive response has been shown to protect against necrosis (Li, G.C. *et al.* 1990; Liu, G.S. *et al.* 1991; Liu, Y. & Downey, J.M. 1992), arrhythmias (Hagar, J.M. *et al.* 1991; Lawson, C.S. & Hearse, D.J. 1994), and post ischemic contractile dysfunction (Banerjee, A. *et al.* 1993; Steenbergen, C. *et al.* 1993). In Murry's experiments, if the ischemia was extended to three hours, there was no difference in infarct size between the pre-treated and control groups, showing that with sustained ischemia, cells must eventually die, irrespective of preconditioning (Murry, C.E. *et al.* 1986).

Soon after the discovery of ischemic preconditioning, a variety of preconditioning stimuli were uncovered, including hypoxia (Cohen, M.V. *et al.* 1995), rapid cardiac pacing (Koning, M.M.G. *et al.* 1996; Vegh, A. *et al.* 1991), heat stress (Walker,

D.M. *et al.* 1993), alcohol (Krenz, M. *et al.* 2001), stretch (Gysembergh, A. *et al.* 1998; Ovize, M. *et al.* 1994), exercise (Domenech, R. *et al.* 2002; Parent De Curzon, O. *et al.* 2001) and various pharmacological agents (Cohen, M.V. *et al.* 2000; Yellon, D.M. *et al.* 1998). The common outcomes of all the above preconditioning stimuli are the reduction in myocardial infarct size, prevention of arrhythmia and improvement in heart function.

However, not all combinations and durations of ischemia and reperfusion were shown to trigger preconditioning phenomenon and protect ischemic and later reperfused myocardium. There appears to be a critical threshold. A single preconditioning stimulus of only one or two minutes of ischemia and reperfusion, prior to the index ischemia, has no protective effect in rabbits (Vanwinkle, D.M. *et al.* 1991), pigs (Schultz, J.E.J. *et al.* 1998). One five-minute cycle of ischemia prior to the index ischemia reduced damage in the dog, yet six and 12 cycles of five-min coronary occlusions produced no protection (Li, G.C. *et al.* 1990). Similar observations were repeated using human cardiac tissue *ex vivo* with similar results (Morris, S.D. & Yellon, D.M. 1997). Another study conducted by Ghosh and co-workers found the loss of protection when the one cycle preconditioning ischemic stimulus was extended to 10 minutes, and it was concluded that repeated occlusions of a coronary artery during percutaneous transluminal coronary angioplasty or occlusions of the ascending aorta during cardiac surgery totalling 10 or more minutes of ischemia, may result inadvertently in loss of protection (Ghosh, S. et al. 2000). It seems more than likely that preconditioning is following a steep dose-response curve. Once a maximal response is achieved, further stimulation has no

additional effect and may in fact reversing its effect or even cause damage. It appears that one cycle of five minutes of ischemia was enough to cause protection. One cycle of two minutes of ischemia was too short to cause protection. One cycle of ten minutes of ischemia was too long to cause protection.

The protocol of three cycles of five minutes intermittent ischemia has been commonly used to demonstrate preconditioning reduction in infarct size in isolated rat heart studies (Fryer, R.M. *et al.* 1999a; Liu, Y. & Downey, J.M. 1992; Yue, Y. *et al.* 2001). In other animal models, it was reported that one cycle of five minutes ischemic preconditioning was not as efficacious in reducing infarct size before a prolonged ischemic insult as three cycles of five minutes intermittent ischemic preconditioning protocol (Fryer, R.M. *et al.* 1999a).

In summary, the above studies suggested that one cycle of preconditioning stimulus less than two minutes and more than ten minutes causes no protection. One cycle of five minutes preconditioning stimulus does cause protection, however, infarct-limiting effect of one cycle of five minutes ischemic preconditioning protocol is not as efficacious as three cycles of five minutes intermittent ischemic preconditioning protocol. If the ischemia after preconditioning stimulus is extended to hours, there was no protection regardless of the preconditioning stimulus, with continuing ischemia, cells must eventually die.

This study applied well established and widely used protocol of ischemic preconditioning in the rat isolated heart (Fryer, R.M. *et al.* 1999b; Liu, Y. & Downey, J.M. 1992; Schultz *et al.* 1995; Schultz *et al.* 1996; Yue, Y. *et al.* 2001).

The isolated heart is one of the most popular experimental models in cardiovascular research. The isolated perfused small mammalian heart represents the optimal compromise in the conflict between the quantity and quality of data that can be achieved from an experimental model versus its clinical relevance-particularly in relation to the modelling of ischemia (Sutherland, F.J. & Hearse, D.J. 2000). At a practical level, the isolated heart from small mammals provides a highly reproducible preparation, which can be studied quickly and in large numbers. It allows a broad range of biochemical, physiological and morphological indices to be measured. These measures can be made without confounding effects of other organs, the systemic circulation, and a host of peripheral factors, such as circulating neuro hormonal factors and circulating fatty acids. More importantly, the isolated heart preparation allows experiments to be continued in the face of events such as infarction-induced loss of pump function and cardiac arrhythmias, which would normally jeopardise the survival of an *in vivo* experiment (Sutherland, F.J. & Hearse, D.J. 2000).

The isolated perfused heart preparation is largely based on adaptations of that originally described by Langendorff in 1895 (Doring, H.J. & 1990). Langendorff succeeded in isolating and perfusing the mammalian heart which continued to beat and pump blood for several hours. The method has become a fundamental tool in pharmacological and physiological research, and during cardiac transplant surgery in preserving the donor heart. The principle of the Langendorff apparatus is to provide the heart with oxygen and nutrients via a perfusate, that is, blood or physiological solutions. The cannulated ascending aorta is attached to a reservoir which contains perfusate. Pressurized perfusate from the reservoir is forced through

the ostia (openings where the coronary arteries are attached to the aortic root) into the coronary bed. This is often termed retrograde perfusion, because the perfusate flows into the aorta toward the heart, rather than out of the left ventricle through the aorta, as blood flows *in vivo* (Sutherland, F.J. & Hearse, D.J. 2000). The leaflets of the aortic valve are forced shut due to the constant hydrostatic pressure (usually in the range of 60-100mmHg, as it is in the *in situ* heart during diastole), and the perfusion solution is directed via the coronary ostia into the coronary arteries, thereby perfusing the entire ventricular mass of the heart and it exits coronary venous circulation via the coronary sinus into the right atrium (Sutherland, F.J. & Hearse, D.J. 2000). Figure 3.1A shows the anatomy of the heart and Figure 3.1B is a diagram showing the coronary ostia. During this time, various instrumentations of the heart can be undertaken. Electrocardiographic recordings allow the detection, identification and quantification of abnormalities of cardiac rhythm. Contractile activity can be assessed by connecting a tension transducer to the ventricular apex and recording muscle shortening, as was done by Langendorff or via a pressure transducer inserted into the left ventricle.

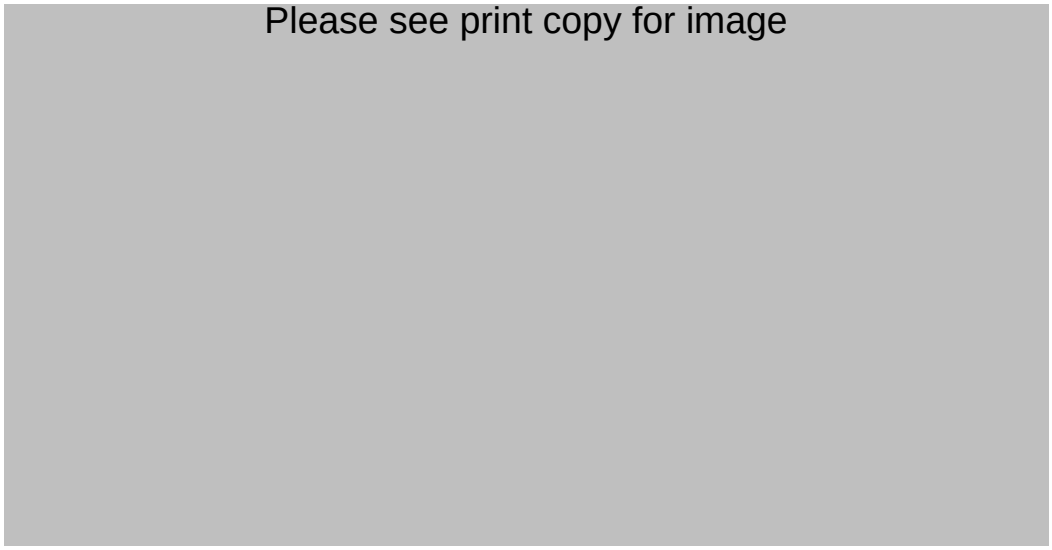


Figure 3.1A Anatomy of the heart (adopted by AD Research, www.adinstruments.com)

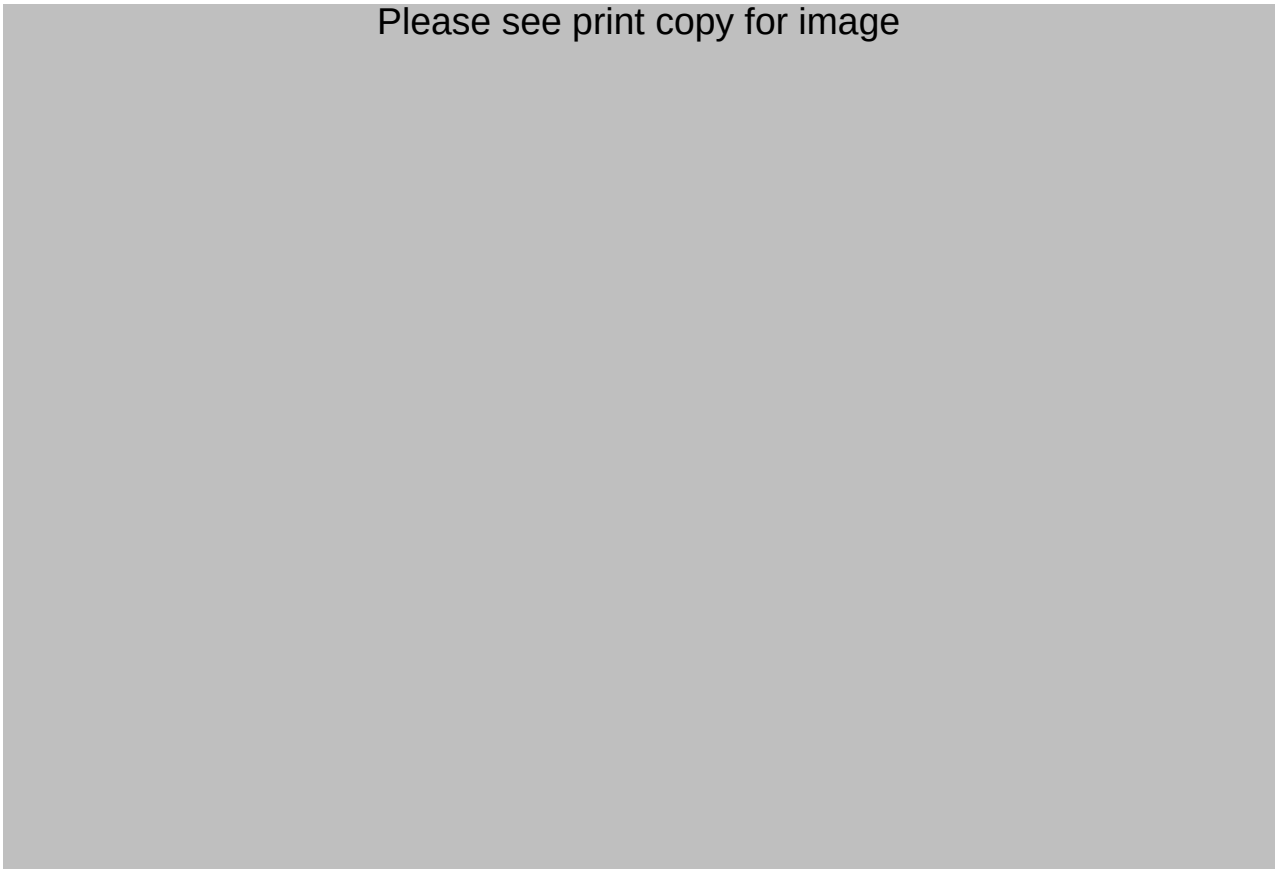


Figure 3.1B Diagram showing origin of right and left coronary ostia and coronary arteries. Adapted source: Fuster V, Hurt's The Heart

However, the Langendorff preparation in the isolated heart does not permit the left ventricle to eject the perfusate (no cardiac output) and is therefore a non-working model.

Rats have been accepted as an appropriate model for learning about human physiology and disease (including heart disease) since the early 1800s. As to selecting the best species for perfusion, although isolated perfusion of large animal hearts such as pigs, monkeys, sheep, dogs and rabbits has been reported, rats are by far the most frequently used and the best characterized isolated heart model (Curtis, M.J. 1998; Galinanes, M. & Hearse, D.J. 1990; Sutherland, F.J. & Hearse, D.J. 2000; Ytrehus, K. 2000). This is probably because of the relative lower cost and less variability, as well as smaller volumes of perfusion fluids and less cumbersome equipment required. Rat models also predominate over other experimental models of ischemia-induced arrhythmias (Curtis, M.J. 1998). Other reasons to choose the rat model include the similarity of their nutritional requirements to those of humans, the fact that rats are mild tempered, relatively straightforward to take care of and to feed with a specialized diet for a long term - which is hard to do with larger animals. Furthermore, this laboratory has established many effects of dietary fats on heart composition and function in rats that will be used for comparison with this study (McLennan, P.L. *et al.* 1985; McLennan, P.L. 1993; McLennan, P.L. & Dallimore, J.A. 1995; McLennan, P.L. 2001).

In the present study, three different types of fat diet groups were chosen; the diet of main interest contained the long-chain (n-3) PUFA, containing EPA and DHA from

fish oil. However, in the literature, both (n-6) PUFA and (n-3) PUFA were claimed to have cardioprotective effects. In order to evaluate the specific role of (n-3) PUFA and not non-specific effects of any PUFA, the (n-6) PUFA diet was designed and both were compared to low PUFA saturated fat rich diet (SF). The SF diet was also designed to evaluate whether the effects of the (n-3) PUFA diet could be attributed to low levels of essential (n-6) PUFA. The oil blends in the (n-3) PUFA diet and the (n-6) PUFA diet were designed to deliver similar total PUFA. The oil blends in the (n-3) PUFA diet and the SF diet were designed to deliver similar low total (n-6) PUFA.

A six week feeding protocol was chosen to ensure that the major myocardial (n-3) PUFA, DHA reached equilibrium within cellular membranes (Owen, A.J. *et al.* 2004). By choosing a six-week period, an unwanted possible source of variation due to a shorter feeding period (resulting in variable degrees of incorporation) was removed. The rationale for the three diet treatments as outlined above and time length is comparable to that for previous studies (Owen, A.J. *et al.* 2004; Pepe, S. & McLennan, P.L. 2002).

3.2 METHODOLOGY

This section describes the animals and dietary protocol, diet preparation, experimental procedures, data acquisition and ischemic preconditioning protocol and statistical procedures.

3.2.1 Animals and dietary protocol

Male Wistar rats (from the Animal Resources Centre, Western Australia) were housed two per cage on a twelve hour reverse light/dark cycle at a temperature 22°C. and were given standard laboratory chow and tap water ad libitum. They were randomized into three experimental groups at nine weeks of age to receive one of three iso-energetic diets containing different types of fat. The fats were predominantly saturated fat, (n-6) PUFA or (n-3) PUFA with other dietary components based on the American Institute of Nutrition AIN93 rat diet (Reeves, P.G. 1997), containing all essential vitamins and minerals but with gelatine as part of the protein source for six weeks. Figure 3.2 provides a schematic representation detailing the groups and treatment regimes, as well as investigations carried out.

3.2.2 Diet preparation

The diet was prepared with 10% fat by weight w/w (22% metabolisable energy) consisting of 7% w/w fish oil (NuMega high DHA tuna fish oil) plus 3%w/w olive oil (Meadow Lea Foods) (n-3 PUFA diet) or 5%w/w sunflower seed oil (Meadow Lea Foods) plus 5%w/w olive oil (n-6 PUFA diet) or 7%w/w beef tallow (Meadow Lea Foods) plus 3% w/w olive oil (SF diet). Gelatine replaced some casein to permit the diets to be mixed wet. Casein as a sole protein source is often considered deficient in the sulphur amino acids methionine or its derived amino acid cysteine. The addition to the food blend of gelatine protein consisting of

approximately 1% methionine overcomes this potential deficiency and there was no evidence of adverse effects on animal growth with these diets.

The diets were then set in trays at 4°C for approximately 20 minutes, sliced into cubes and kept frozen at –20°C until use. A small amount of food colouring was added to each diet for identification purposes. Rats were allowed free access to both food and water. Rats and their diet were checked every two days to ensure the adequacy of the diet. At the end of six weeks, animals were weighed. The diet composition is shown in table 3.1.

Table 3.1 Nutrient composition of rat diets

Ingredient	Amount	Source
Cornstarch	565g	commercially available
Sucrose	100g	commercially available
Casein	90g	NZ Milk Products Ltd.Wellington NZ
Fibre	50g	commercially available
Vitamin	10g	MP Biomedicals Inc.,Seven Hills, Australia
Mineral	35g	MP Biomedicals Inc.,Seven Hills, Australia
Oil	100g	commercially available
Gelatine	50g	commercially available
Fat*	100g	variable sources
Total	1000g	
Water	400mL	

Type of fat is varied in the three diets.

3.2.3 Experimental procedures, data acquisition and ischemic preconditioning protocol

Animal care and experiments were conducted with the approval of the University of Wollongong Animal Care and Ethics Committee. The investigation conforms to the guidelines of the National Health and Medical Research Council, Australia, Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC 2004).

Prior to the experiment, the height of the perfusion reservoir was set at 1.02m above the aortic cannula to provide perfusion pressure of 75mmHg (1 mmHg = 1.36 cmH_2O). Pressure was calibrated by applying a known pressure using a mercury sphygmomanometer (See Figure 3.7A). The pressure transducer (Cobe), which converts the pressure signal into an electrical signal, was connected to a calibrated Bioamplifier . Rats were euthanased by heart removal under anaethesia (intraperitoneal injection of sodium pentobarbital at 60mg per kilogram of body weight).

Anaesthesia was checked by absence of the foot withdrawal reflex. Once the rat was anesthetized, the rat was positioned supine to excise the heart. Briefly, the diaphragm was accessed by a transabdominal incision and was cut carefully to expose the thoracic cavity. The thorax was opened by bilateral incision along the lower margin of the last to first ribs; the thoracic cage was then reflected towards the animal's head, exposing the heart. The heart then was gently cradled between fingers (essential to avoid contusion injury) and was lifted slightly before incising the aorta, vena cava and pulmonary vessels. During the dissection of the heart, it was important to leave enough length of aorta to connect to the perfusion apparatus. After the dissection, the heart was immediately placed in a beaker containing ice-

cold saline in order to rinse it of blood, temporarily arresting its beating. While still submerged in cold saline, the aorta was then gently eased over the end of the fluid filled cannula, taking care not to insert the cannula too far into the aorta since this would occlude the coronary ostia or damage the aortic valve.

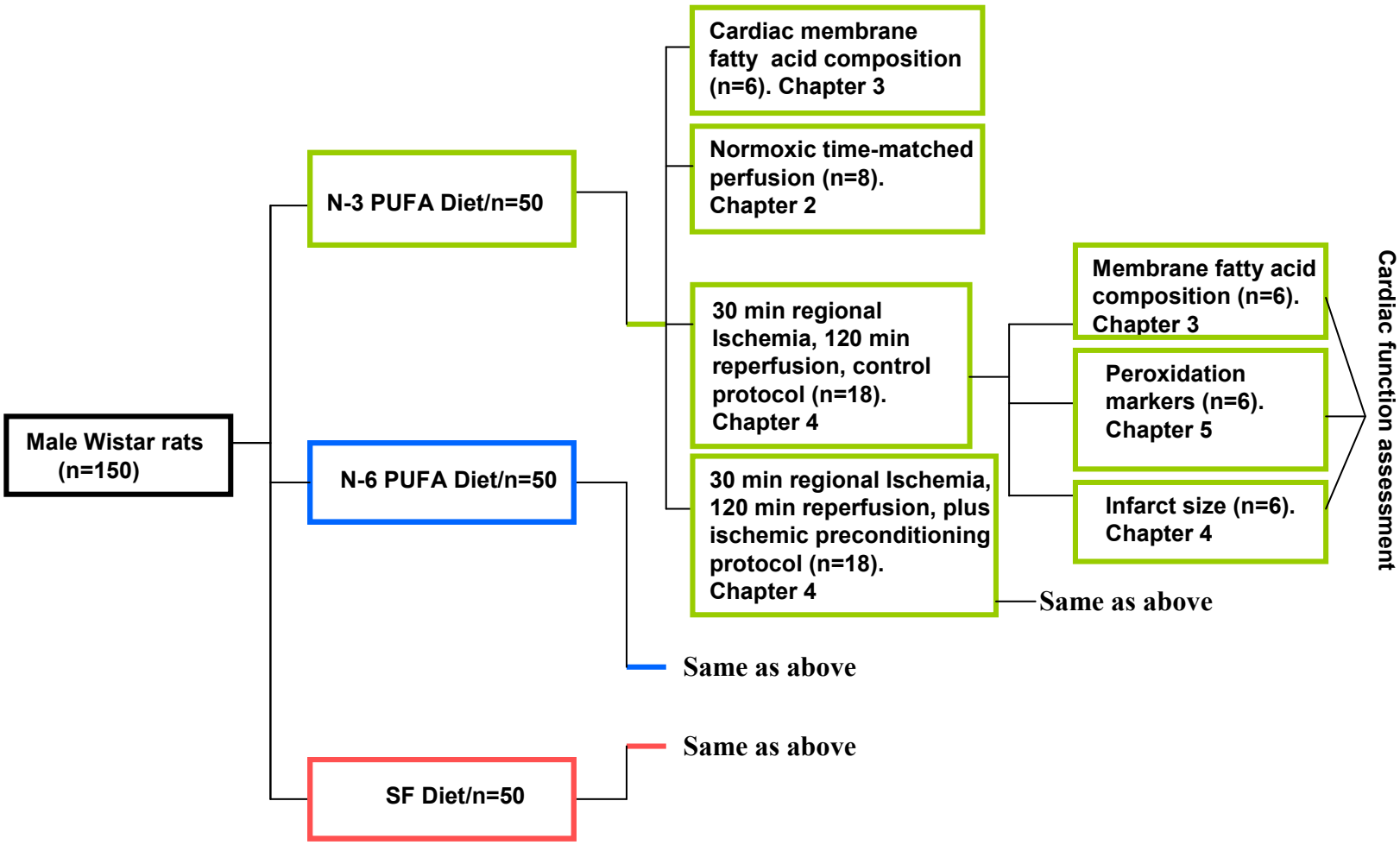


Figure 3.2 Dietary groups and investigations

A ligature was rapidly tied around the aorta. During this process, the heart was held gently, taking care to avoid stretching or tearing of the aortic wall. After the cannulation, the heart was attached to a Langendorff perfusion apparatus. Figure 3.3 shows schematic diagram of apparatus utilized to acquire data.

After the heart was rapidly attached to the perfusion apparatus, perfusion was initiated immediately with filtered and heated (37°C) Krebs-Henseleit bicarbonate buffer (Sutherland, F.J. & Hearse, D.J. 2000). This perfusion fluid, which mimics the key ionic content of blood or plasma, has a pH of 7.4 at 37°C (See Appendix 3.1 on description of how to make the buffer). Central to the survival of any perfused organ is continuous provision of oxygen in quantities sufficient to support normal metabolism. In this experiment, oxygen was provided by gassing the perfusion fluid with high concentration of oxygen, 95% O_2 and 5% CO_2 (the CO_2 was required to achieve the correct pH 7.4 in the buffer) at 37°C (Sutherland, F.J. & Hearse, D.J. 2000). The myocardial temperature was maintained at 37°C throughout the experiment by a thermostatically controlled water-jacketed system in which all glass reservoirs and the heart perfusion chamber were surrounded by rapidly flowing water at 37°C. The heart was perfused in the Langendorff mode under constant pressure of 75 mmHg. The value of systolic *in situ* perfusion pressure for rat hearts ranges from 70~90mmHg (Doring, H.J. & 1990). However, the recommended perfusion pressure for the isolated heart is around 70mmHg. This is on account of lower flow resistance and tissue oedema when using saline solution (Doring, H.J. & 1990).

During the first 30 minutes of perfusion, the left atrium was opened by making a small incision, and a plastic thin walled balloon connected to a pressure transducer (Cobe) was introduced into the left ventricle via the mitral valve to measure left ventricular pressure. The signal obtained from the heart was recorded on computer as a digital input at a predetermined sampling frequency using the software, LabView for Windows (National Instruments). The balloon was as thin as possible, flexible and compatible with biological tissues. The standard latex balloon which is commonly used by researchers for Langendorff heart preparations is limited in its ability to meet these criteria (Curtis, M.J. *et al.* 1986; Sutherland, F.J. *et al.* 2003).

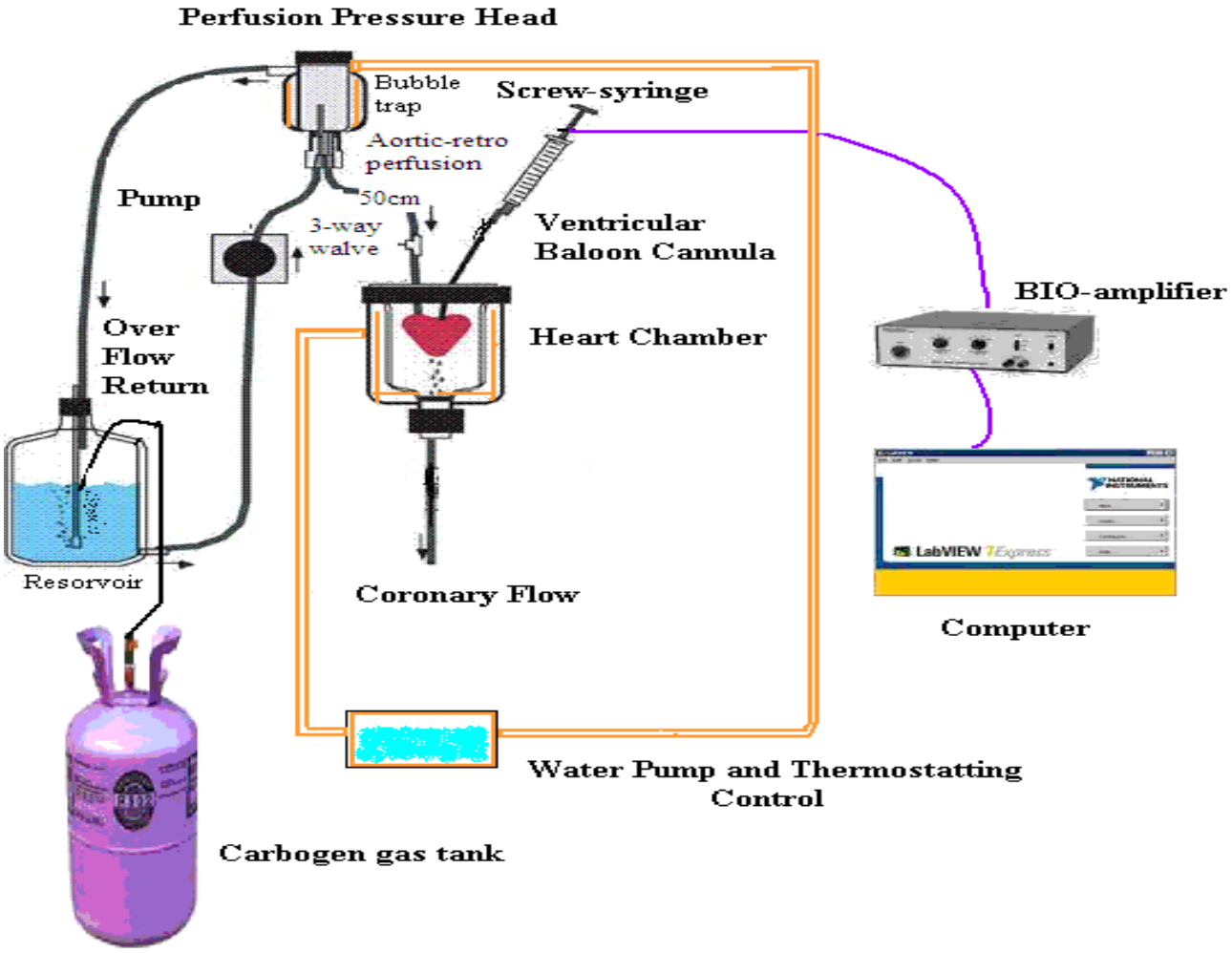


Figure 3.3 Schematic diagram of apparatus utilized to acquire data

For this reason, this study utilized homemade balloons made from cling film (see Appendix 3.2 for description of making a "balloon"). After the balloon was inserted into the left ventricle, the balloon volume was adjusted by inflating the balloon with water using a water filled screw-adjustable syringe to set an end diastolic pressure of 6-8mmHg (similar to *in situ* heart during diastole). An electrocardiogram (ECG) was recorded continuously with subdermal needle electrodes placed at the surface of ventricular apex and the aortic stump. Data obtained from the pressure transducer connected to the heart was converted from millivolts to mmHg mercury through the biological amplifier and recorded via the data acquisition and processing program Labview for Windows (National Instrument). Data points were recorded at 200Hz.

Hemodynamic measures of systolic peak pressure, end diastolic pressure, maximum contraction and relaxation rate were recorded through a water filled balloon catheter attached to the pressure amplifier and constantly monitored using the program Labview for Windows. Coronary flow was recorded every five minutes by collecting and measuring the effluent that dripped from the heart. Heart rate was obtained from the intraventricular pulse. Rate pressure product (indicative measurement of oxygen consumption) (Nelson, R.R. *et al.* 1974) was calculated by multiplying heart rate by systolic pressure. The left ventricular developed pressure was calculated by subtracting end diastolic pressure from left ventricular systolic pressure.

Experiments commenced with 30 minutes equilibration, after which a suture was quickly threaded under the left ventricular descending coronary artery located between the base of pulmonary artery and left atrium. Regional ischemia in this experiment was defined as the cessation of blood flow produced by occluding the left anterior ventricular descending coronary artery for 30 minutes. Reperfusion for 120 minutes was induced by releasing the occluding ligature. In order to observe independently the effect of preconditioning, separate groups of hearts were subjected to an ischemic preconditioning protocol prior to 30 minutes ischemia. This consisted of three cycles of five minutes global ischemia, each followed by five minutes reperfusion before the onset of 30 minutes regional ischemia, then 120 minutes reperfusion. Global ischemia in this experiment was defined as a complete cessation of blood flow to whole heart. Global ischemia was induced by closing the 3-way valve above the heart attached to the perfusion apparatus and stopping all flow through the coronary arteries. Figure 3.4 illustrates the experimental protocol.

The primary endpoint of this experiment was infarct size, which is considered the "gold standard" of ischemic preconditioning (Przyklenk, K. & Kloner, R.A. 1998). For measuring infarct size, the left anterior descending coronary artery was reoccluded after 120 minutes reperfusion, and then the coronary arteries were perfused with Evans Blue dye. The ventricular wall receiving unimpeded coronary flow becomes stained and represents the non-ischemic myocardium. The region of myocardium normally supplied by the occluded coronary artery remains unstained and this region represents the ischemic zone at risk of infarction. Afterwards, hearts were set aside to measure infarct size (details in Chapter Five). Some groups of

hearts were used immediately to measure oxidation products (Chapter Six). Some groups of hearts were rapidly placed in cold Krebs Henseleit buffer, blotted dried, weighed and placed in -80°C for later analysis (Chapter Five, Chapter Six).

Statistical procedures

Statistical analysis was conducted using SPSS 15.0 for Windows student version or Statistix. Comparison was made between dietary groups and treatment groups with two-way analysis of variance (ANOVA), with Tukey's post hoc test for comparison of individual means. Two-way ANOVA was used to determine any differences between groups at individual time-points, using diet and ischemic preconditioning as the main effect and diet × preconditioning interaction. Values were considered significant if $p<0.05$.

3.3 RESULTS

3.3.1 Effects of diet on rat characteristics

Rats were weighed at the start of diet treatments (week zero) and before being sacrificed (week six). Weight gain was then calculated as weight at week six minus initial weight. Heart weight and tibia length were measured after sacrifice or at the end of the experiment. Heart weight to body weight ratio was calculated.

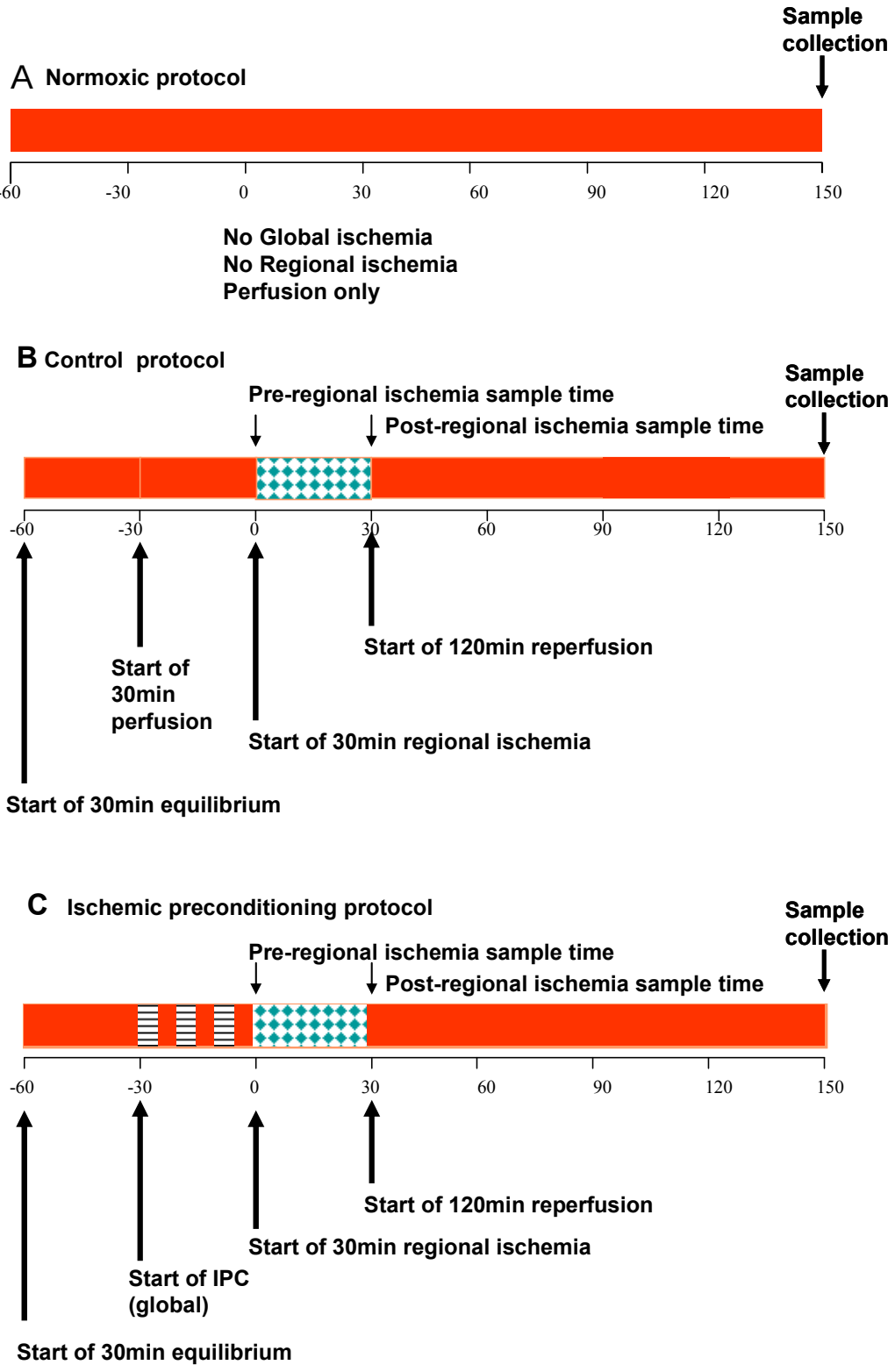


Figure 3.4 Experimental protocols: perfusion, global ischemia, regional ischemia. A: 210 minutes of perfusion with no ischemia. B: 30 minutes of equilibrium 30 minutes of regional ischemia and 120 minutes reperfusion. C: Preconditioning protocol-hearts, three episodes of global ischemia for five minutes, each separated with reperfusion for duration of five minutes, followed by regional ischemia for 30 minutes and reperfusion for 120 minutes after the 30 minutes equilibrium period.

There were no significant differences in body weight between any of the groups at week zero or at time of sacrifice following six weeks of dietary manipulation. There was no effect of diet on weight gain, calculated as weight at week six minus initial weight, or body size as in indicated by tibia length. No dietary differences in heart size were evident as determined by heart mass or heart weight to body weight ratio (Table 3.2).

Table 3.2 Body weights and heart weights of diet treated rats

Diet	SF	(n-6) PUFA	(n-3) PUFA
n	50	50	50
Body weight (g) wk=0; 18 wk old	343±13	344±11	347±10
Body weight (g)wk= 6; 24 wk old	474±6	470±11	473±8
Weight gain(g)	131±10	126±8	125±9
Heart weight(g)*	1.46±0.1	1.43±0.1	1.42±0.1
Tibia length(mm) (n=18)	43.5±0.3	43.4±0.2	44.9±0.5
Heart weight/body weight(mg/g)	3.1±0.2	3.0±0.4	3.0±0.2

Values are means ± SEM, * Ventricle weight measured after ischemia-reperfusion protocol with atria and major vessels removed.

3.3.2 Preliminary observations of heart function with perfusion

Measurement of heart function

In a normally beating heart, the lowest pressure recorded between beats represents the left ventricular end diastolic pressure. End diastolic pressure was adjusted to

6~8mmHg at commencement of perfusion. The peak pressure represents left ventricular systolic pressure. The difference between systolic pressure and end diastolic pressure is developed pressure. Sample traces are shown from a single representative experiment using the heart from a saturated fat fed diet, subjected to ischemic preconditioning, 30 minutes regional ischemia and 120 minutes reperfusion. Pressure readings were taken as the mean over five seconds. Beating hearts during normal perfusion achieved up to 100mmHg peak systolic pressure (Figure 3.6) and the calculated developed pressure was around 92mmHg (Figure 3.6). Peak dP/dt was derived from the ventricular pressure tracings (Figure 3.5). The maximal rate of pressure rise (+dP/dt) during ventricular contraction represents how fast a heart develops pressure within each beat (the maximum rate of pressure rise during isovolumic contraction). Maximum rate of relaxation (-dP/dt) shows how fast the heart relaxes within each beat (the maximum rate of pressure fall during isovolumic relaxation).

After the pressure calibration, the beating hearts developed 100mmHg peak systolic pressure and end diastolic pressure stabilized at 6~8mmHg or higher. The black curves where lines were superimposed showed fluctuations in ventricular pressure representing the underlying heart rate (Figure 3.7A). When global ischemia was induced, pressure gradually dropped toward zero (Figure 3.7B, C). Arrhythmias often occurred at the start of reperfusion but systolic pressure gradually increased and stabilized up to 80mmHg (Figure 3.7C). Regional ischemia gradually reduced the systolic pressure, while end diastolic pressure increased (Figure 3.7E). During this period, arrhythmias often occurred (Figure 3.7F). Ventricular Tachycardia was

identified as four or more consecutive premature beats and fibrillation was manifested as a rapid, chaotic, and asynchronous contraction of the left ventricle (Figure 3.7F). Most hearts spontaneously recovered from ventricular fibrillation during regional ischemia (Figure 3.G) with systolic function restored. Hearts fibrillating for more than two minutes were manually defibrillated by flicking with a finger in a manner simulating a primordial thump that might be used in human cardiac resuscitation in the absence of an electrical defibrillator. Few experiments discarded because heart did not revert back to normal. Reperfusion restored the developed pressure which was then maintained until the end of the three-hour experiment (Figure 3.7H).

Preliminary data of the diet effects on heart function

Langendorff hearts were set up and perfused over a period of three hours to establish stability of the preparation without any ischemia intervention (Dietary differences in heart function are described in Chapter Five). Left ventricular pressure was maintained well during three hours of perfusion if no ischemia was imposed.

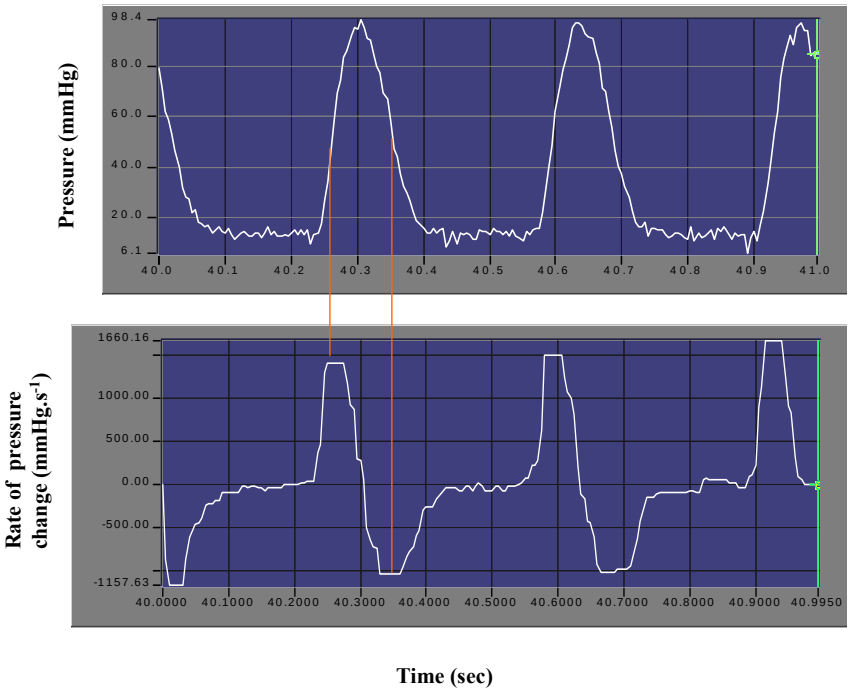


Figure 3.5 Example data trace from a typical rat heart showing the intraventricular pressure (upper panel) and the rate of positive and negative pressure change derived from the pressure data. The lines connection upper and lower traces indicate the points of maximum rate of contraction (+dp/dt max) and relaxation (-dp/dt max) on both traces (1sec).

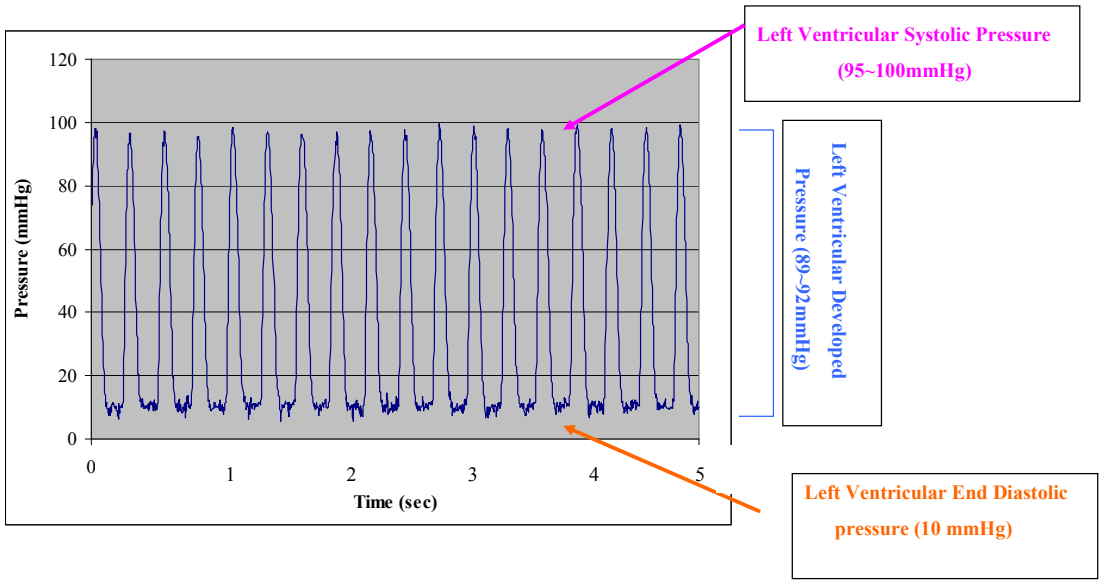


Figure 3.6 Example data trace from a typical rat heart showing left ventricular systolic pressure, developed pressure and end diastolic pressure (4 samples/sec)

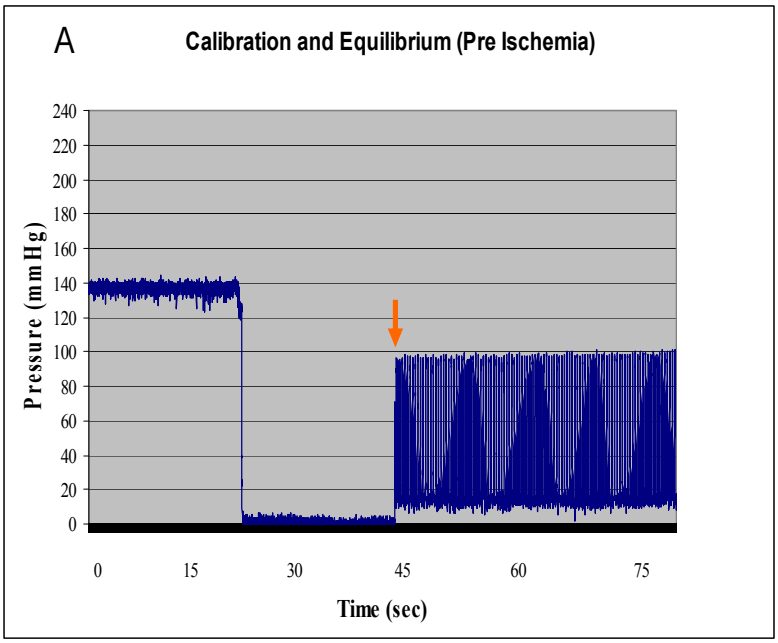


Figure 3.7A Example data trace from typical rat heart showing intraventricular pressure. The early part of the trace shows the calibration signal of known pressure applied to the transducer. The arrow indicates the onset of recording from the intraventricular balloon.

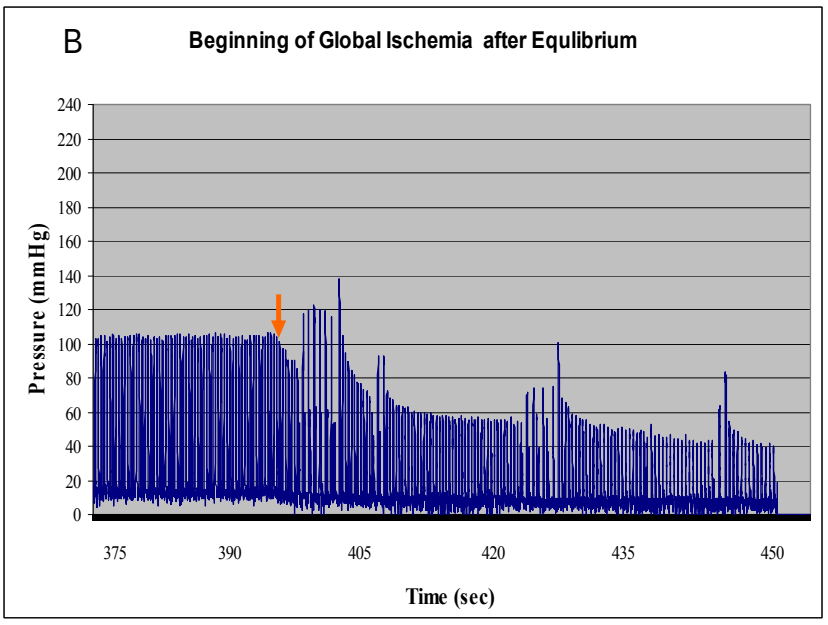


Figure 3.7B Example data trace from typical rat heart showing intraventricular pressure recording during equilibrium and during global ischemic preconditioning. The arrow indicates the onset of global ischemia.

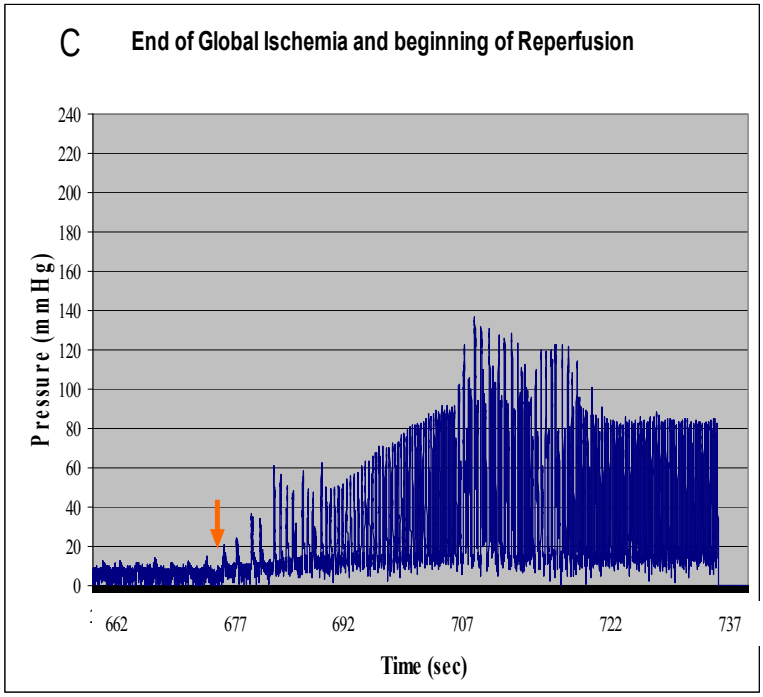


Figure 3.7C Example data trace from a typical rat heart showing intraventricular pressure at the end of 5min period of ischemic preconditioning global ischemia and in the early stages of reflow. The arrow indicates the onset of reflow into the coronary arteries.

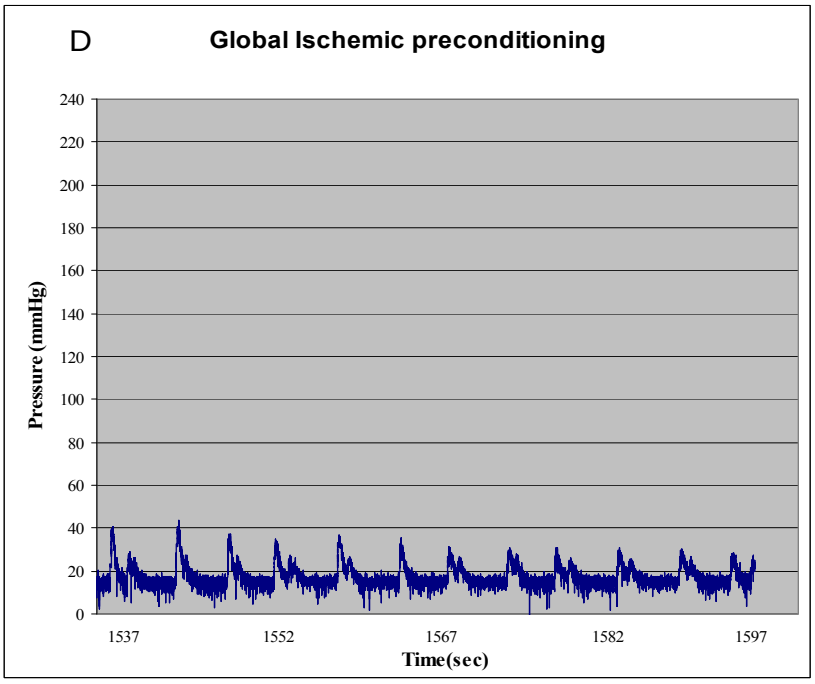


Figure 3.7D Example data trace from a typical rat heart showing a 60-second excerpt during a global ischemic preconditioning episode. Heart has slowed almost to a stop.

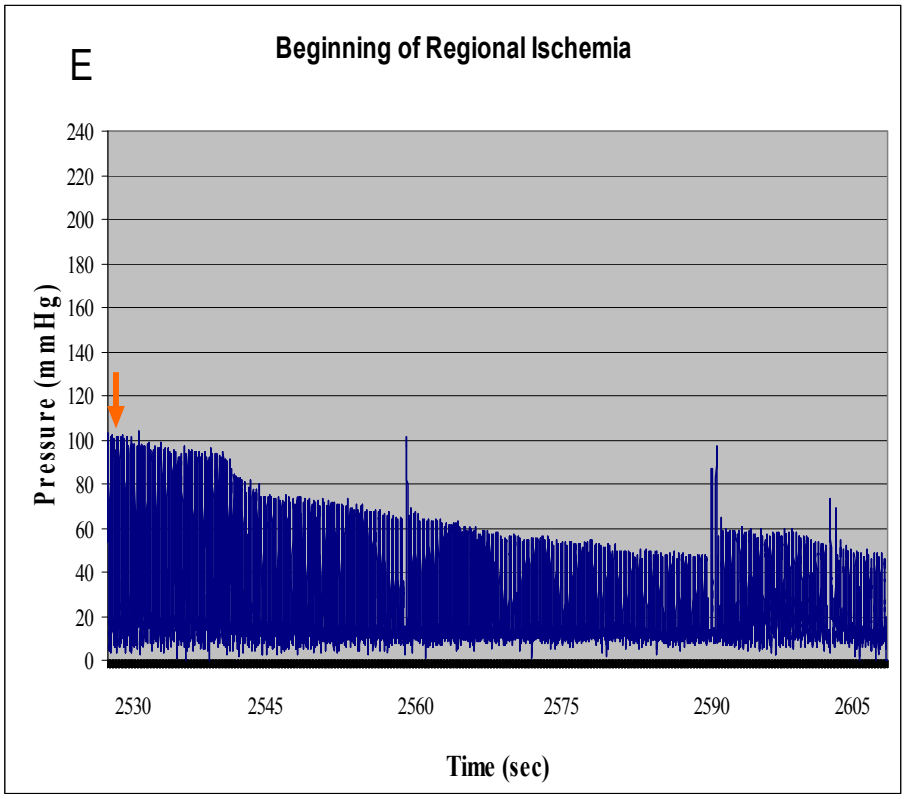


Figure 3.7E Example data trace from a typical rat heart showing intraventricular pressure during the early stages of 30-minute regional ischemia. The arrow indicates the time of coronary artery occlusion, showing rapid decline in developed pressure and gradual rise in end diastolic pressure.

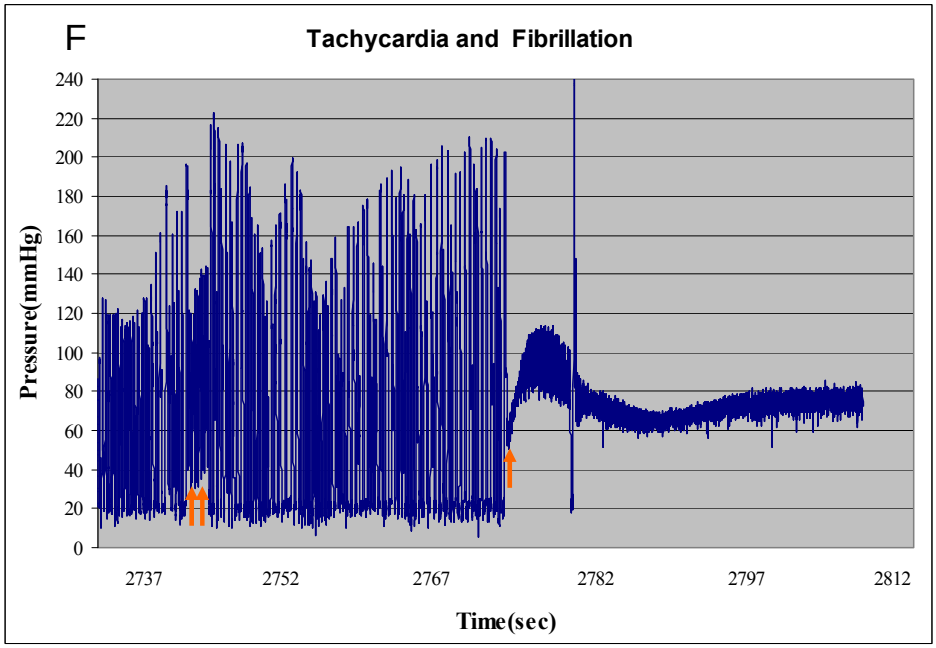


Figure 3.7F Example data trace from a typical rat heart showing intraventricular pressure during regional ischemia with frequent ventricular arrhythmias. The double arrow indicates a period of ventricular tachycardia; the single arrow indicates the onset of an episode of ventricular fibrillation.

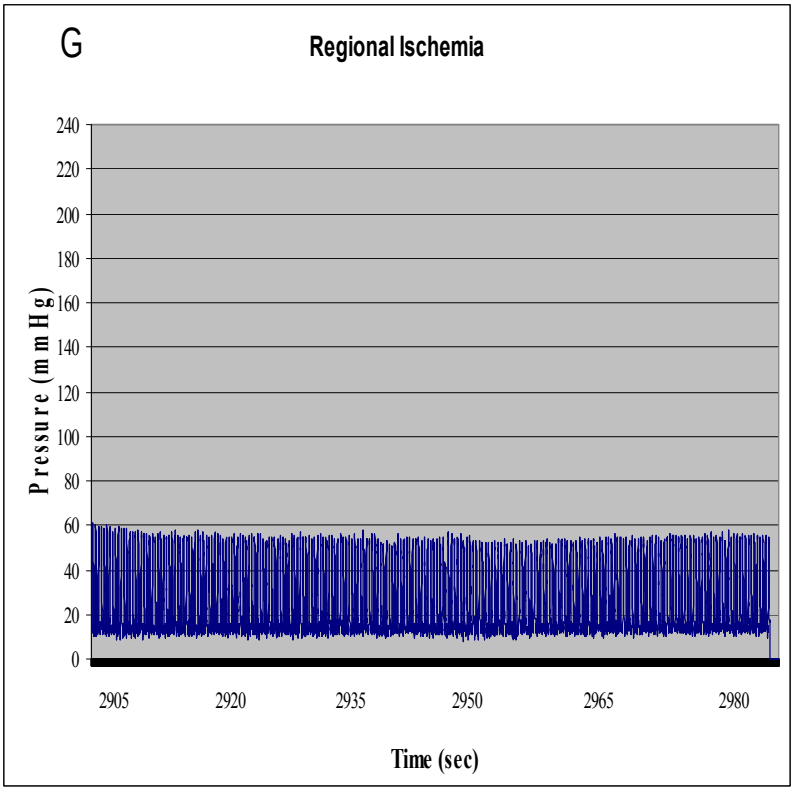


Figure 3.7G Example data trace from a typical rat heart showing intraventricular pressure midway through the 30-minute period of regional ischemia, showing depressed developed pressure and elevated end diastolic pressure.

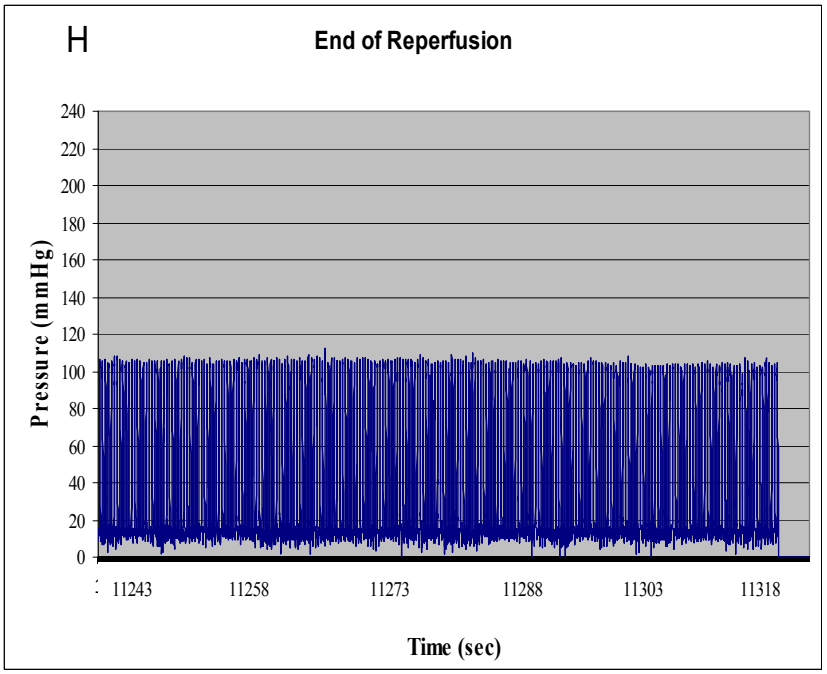


Figure 3.7H Example data trace from a typical rat heart showing restored developed pressure and only partially restored end diastolic pressure during 120 minutes reperfusion after 30 minutes regional ischemia.

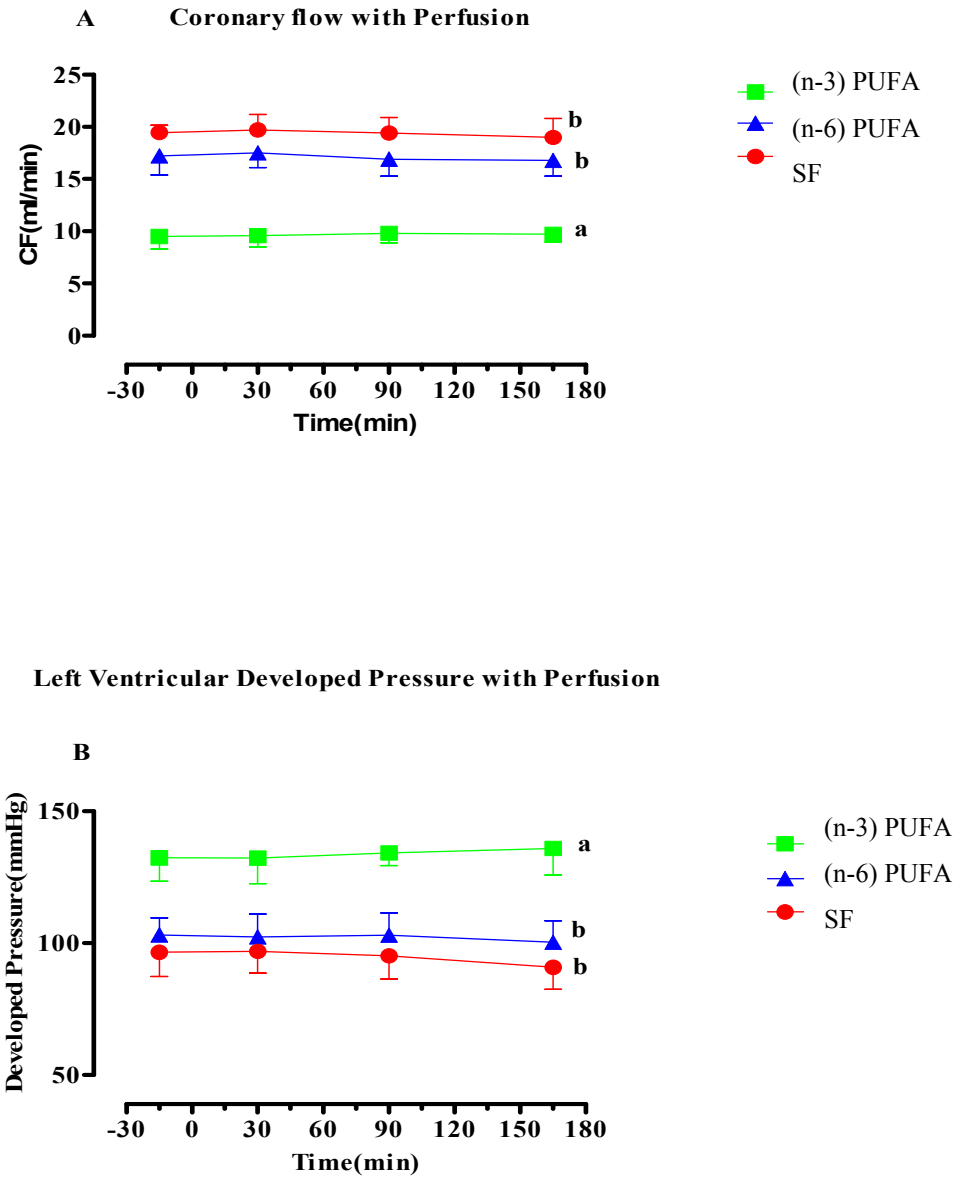


Figure 3.8. Left ventricular developed pressure at various time points in SF, (n-6) PUFA or (n-3) PUFA isolated perfused hearts during 210-minute continuous perfusion. Values are means ± SEM, n=6~9. Within diet groups without a common letter differ, $P < 0.01$.

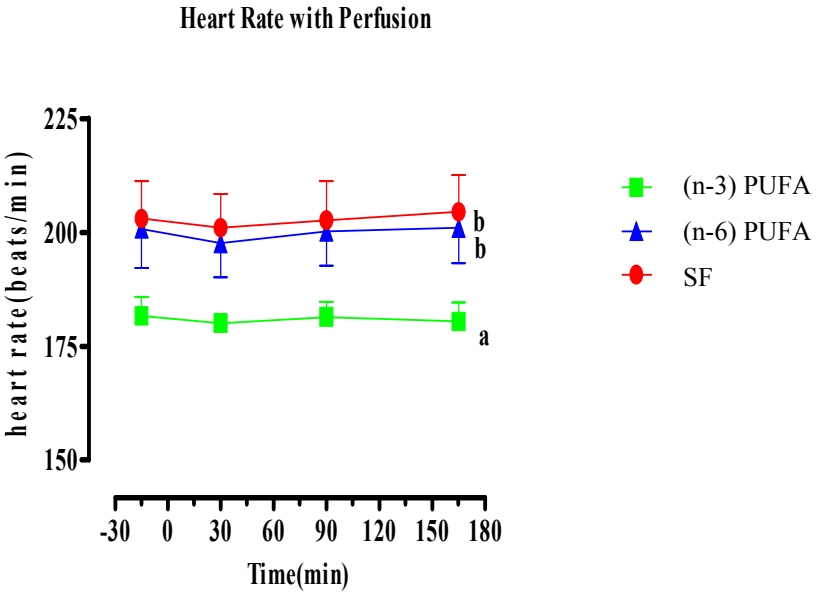


Figure 3.9 Spontaneous heart rate at various time points in SF, (n-6) PUFA or (n-3) PUFA isolated perfused hearts during 210-minute continuous perfusion. Values are means ± SEM, n=6-9. Within diet groups without a common letter differ, $P < 0.01$.

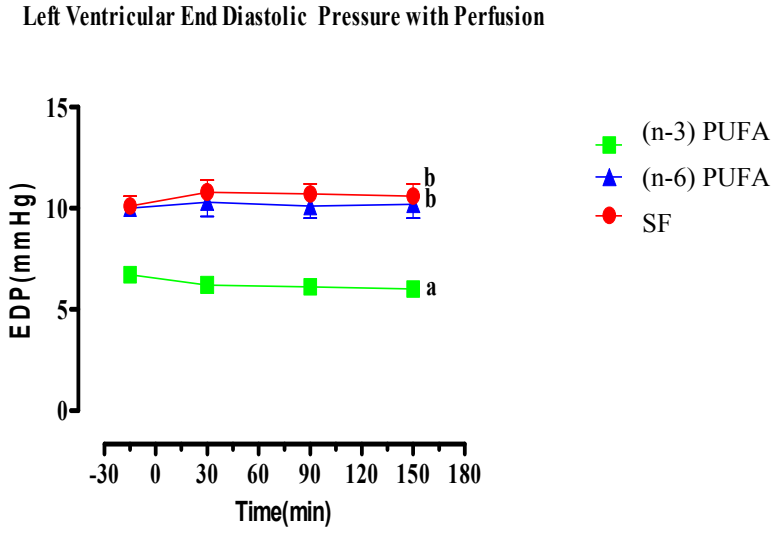


Figure 3.10 Left ventricular end diastolic pressure at various time points in SF, (n-6) PUFA or (n-3) PUFA isolated perfused hearts during 210-minute continuous perfusion. Values are means ± SEM, n=6-9. Within diet groups without a common letter differ, $P < 0.01$.

During perfusion under the constant pressure head of 75mmHg, the (n-3) PUFA hearts exhibited significantly lower coronary flow ($P<0.001$) (Figure 3.8a), spontaneous heart rate that was ≥20 beats.min^{-1} lower ($P<0.01$) (Figure 3.9) and developed pressure that was ≥30 mmHg greater (Figure 3.8b) ($P<0.01$) than in the saturated fat and (n-6) PUFA hearts. These heart functions were not significantly different between SF and (n-6) PUFA hearts.

There was no significant difference in rate pressure product between diets, representing similar cardiac work throughout (data not shown). The end diastolic pressure (Figure 3.9), which was initially set at 6-8 mmHg in all hearts, was significantly lower in the (n-3) PUFA hearts ($P<0.01$) than in SF and (n-6) PUFA hearts at the end of the equilibration period.

3.4 DISCUSSION

The animal growth data indicate that the diets fabricated for this experiment provided adequate nutrition for rats. Rats used in this study were mature (9 weeks old) at commencement of the experiment and managed to gain in excess of 3g/day over the duration of the study.

The experimental set up was stable throughout the perfusion period in the absence of any ischemia. Regular beating of the isolated rat hearts (≈200beats/min) remained throughout the whole perfusion period until the manual cessation of

perfusation. The average heart rate for rats *in vivo* is 320~480beats/min (Baker, H.J. *et al.* 1979). The isolated heart was not under the influence of circulating neurohumoral factors, however, the heart can respond to environmental changes at the level of intracardiac factors. For example, cells of the sinoatrial node respond to mechanical stretching by accelerated pacemaker activity, and generation of the cardiac rhythm depends on ionic balance in pacemaker cells. The model of Langendorff-isolated heart simulates its functioning under physiological conditions. The ionic composition of Krebs--Henseleit solution used for perfusion, is similar to that of the blood, and pH, temperature and pressure approximate physiological levels. At the same time, these experimental conditions affect metabolism of the isolated heart and heart rate parameters. The slower heart rate in isolated heart compared to in *vivo* heart was probably due lack of sympathetic drive needed to support the *in vivo* cardiac output. The isolated heart rate in this study is comparable to other studies using the rat model (Allibardi, S. *et al.* 1999; Tian, R. & Ingwall, J.S. 1996).

The heart function did not change significantly during normoxic perfusion. However, both global ischemia and regional ischemia brought changes in heart function. During global ischemia (used for preconditioning) the perfusate delivery to the whole heart was stopped. Left ventricular pressure decreased abruptly toward zero. Regional ischemia, induced by occlusion of the left anterior descending coronary artery, stopped delivery of the perfusate to only part of the heart; left ventricular developed pressure was only partially reduced, reflecting continual perfusion of a major portion of the heart muscle. This rapid decrease in contractility

is the major adaptation of the myocytes to the decrease in the oxygen supply. After the onset of ischemia, there is an energy imbalance. The high-energy phosphate, particularly phosphocreatine, which protects the level of adenosine 5`-triphosphate (ATP) compounds, rapidly declines with an increase in intracellular inorganic phosphate. This change is one of the major signals to cause the down-regulation of contraction, and the decline in energy status is the main signal to the increase in anaerobic glycolysis. The major beneficial consequence of such anaerobic glycolysis is the increase in production of glycolytic ATP, which is however inadequate to replace the total energy demands of the ischemic contracting myocardium. Intracellular acidosis develops rapidly and decreases contractility because protons displace calcium from binding sites on the contractile filaments (Katz, A.M. & Hecht, H.H. 1969). The increase in neutral lactate in ischemia also decreases contractile activity in the ischemic zone (Tennant, R. 1935) and decreases action potential duration (Wissner, S.B. 1974). Thus, the ischemic myocardium can survive for as long as 15 minutes of severe ischemia via a combination of inhibition of contraction and initiation of anaerobic glycolysis (Kloner, R.A. & Jennings, R.B. 2001).

The occurrence of ventricle arrhythmias during ischemia is partially explained by fatty acid metabolism. Fatty acid metabolism is the cellular trigger which induces the accumulation of arrhythmogenic compounds, mainly long chain acylcarnitine. The actual cause of arrhythmia may be the presence of a non-responding ischemic zone which impairs the natural route of the depolarization wave. Also, the deprivation of blood flow and oxygen during ischemia causes fatty acid oxidation.

This leads to an accumulation of lipid metabolites such as intracellular free fatty acids and acyl-CoA. This increase in metabolites is thought to inhibit different aspects of membrane function, such as the mitochondrial translocase, the sodium pump, and phospholipid cycles. The accumulation of calcium within the ischemic cell activates phospholipases, which breaks down membrane phospholipids. High concentrations of the breakdown products accumulate to form micelles, which may act as detergents on the membranes. Such events may partially explain ischemic ventricular arrhythmias (Opie, L.H. 2004).

Ventricular tachycardia was defined as four or more premature beats of similar morphology (Pepe & McLennan, P.L. 1996). In ventricular tachycardia, there is inadequate time for complete diastolic filling. Ischemia also predisposes the heart to the development of to a totally disorganized ventricular rhythm, termed "ventricular fibrillation". On reperfusion, there was rapid recovery of mechanical function and reversal of the metabolic abnormalities. Predictably, ventricular arrhythmias occurred very soon after the reperfusion because of excess intracellular calcium cycling and formation of free radicals. In high concentrations, free radicals can damage the sarcoplasmic reticulum to cause calcium overload, thereby eliciting calcium-dependent arrhythmias (Xu, K.Y. *et al.* 1997). This study observed an increase in end diastolic pressure during ischemia. The increase in end diastolic pressure can be explained by the increased wall tension and stiffness in the ventricles also caused by calcium overload in ischemia (Kihara, Y. *et al.* 1989).

The preliminary data from this method development suggest that hearts from rats fed fish oil have a lower heart rate, greater developed pressure and lower end diastolic pressure, which may contribute to the cardioprotective effect of (n-3) PUFA. This is discussed in detail in Chapter Four in the context of responses to ischemia and reperfusion.

CHAPTER 4

THE MEMBRANE FATTY ACID COMPOSITION OF THE RAT HEART

4.1 INTRODUCTION

This section briefly describes diet and membrane fats, fatty acids, and fatty acids oxidation.

Composition of diet and membrane fats

Fats and lipids take many forms. Dietary fatty acids are obtained mainly as triacylglycerols or phospholipids. In the human body, fats are stored as triacylglycerols or exist as structural components of cells, mainly in the form of phospholipids, making up about 80% of cell membranes.

Extensive research has shown that dietary fatty acids can influence the relative fatty acid concentrations in heart tissue in rats (Awad, A.B. & Chattopadhyay, J.P. 1983; Ayalew-Pervanchon, A. *et al.* 2007; Charnock, J.S. *et al.* 1983; Charnock, J.S. *et al.* 1986; Demaison, L. *et al.* 2001), pigs (Novak, E.M. & Innis, S.M. 2006), monkeys (McLennan, P.L. *et al.* 1993), and humans (Harris, W.S. *et al.* 2004; Metcalf, R.G. *et al.* 2007). Although all tissues vary in their fatty acid composition in different ways, these studies consistently demonstrate that (n-3) PUFA feeding increases the incorporation of (n-3) PUFA, particularly DHA, in the heart tissue.

For example, Charnock *et al.* (Charnock, J.S. *et al.* 1986) studied the fatty acid composition of rat heart phospholipids after long- term feeding of diets supplemented with sunflower seed oil, or tuna fish oil. Apart from the occurrence of small changes in the relative proportions of palmitic and stearic acid and in the ratio of total saturates to total unsaturates, the most crucial changes were in the relative proportions of the n-3 and n-6 polyunsaturated fatty acids such as, linoleic acid, arachidonic acid, EPA and DHA. The main effect of tuna fish oil, which is rich in DHA, is an increase in the proportion of DHA rather than EPA in the cardiac phospholipids, which is compensated for by a significant decrease in arachidonic acid. Similarly, Metcalf *et al.* (Metcalf, R.G. *et al.* 2007) examined the incorporation of (n-3) PUFA into human myocardial membrane phospholipids during supplementation with fish oil and flaxseed oil. Although feeding flaxseed oil supplementation yielded a small increase in atrial EPA but not DHA, the difference was not significant relative to untreated comparator level. There was no effect of duration of flaxseed oil treatment on any of the phospholipid fatty acid examined. This is reflected in a failure in effect on cellular function. Feeding dietary fish oil for 31 days resulted in a significant increase in EPA and DHA in cardiac phospholipids, which was compensated by a significant decrease in arachidonic acid. Furthermore, although EPA and DHA were present in equal proportions in the diet, the amount of DHA accumulated in atrial phospholipids was greater than EPA (Metcalf, R.G. *et al.* 2007).,This greater accumulation of DHA over EPA is also found in animal studies, even where animals are fed EPA rich oil or purified EPA (McLennan, P.L. *et al.* 1996). In conclusion, feeding fish oil results in a significant

increase of (n-3) PUFA in cardiac phospholipids with DHA being the major component, irrespective of the balance of EPA and DHA in the diet.

Many studies demonstrate that cardiac function is affected by the composition of myocardial membrane fatty acids. This may be a consequence of many changes in intracellular and membrane events, such as ion flux, respiratory electron transport, carrier-mediated transport, membrane-bound enzyme activity, receptor function, intracellular lipid-based second messengers, and eicosanoid synthesis (Demaison, L. *et al.* 2001; Hulbert, A.J. & Else, P.L. 1999; Pepe, S. *et al.* 1999). For example, Pepe *et al.* (Pepe, S. *et al.* 1999) demonstrated an increase in mitochondrial Ca^{2+} in hearts of rats fed saturated fat diets, and a reduction in activation of Ca^{2+}-dependent pyruvate dehydrogenase at rest and after stimulation by catecholamines in hearts of rats fed (n-3) PUFA diet. The activation of cardiac mitochondrial dehydrogenases, including pyruvate dehydrogenase by Ca^{2+} is considered to lead to the maintenance of nicotinamide adenine dinucleotide hydrogenases/nicotinamide adenine dinucleotide (NADH/NAD ratio) and the proton-motive force required for oxidative phosphorylation in the face of increased energy demand (Brandes, R. & Bers, D.M. 1997; Hansford, R.G. 1987). Therefore, there is evidence to suggest many intracellular functions especially involving calcium handling, are altered when membrane fatty acids are altered.

The present study aimed to modify the fatty acid composition of the membrane phospholipids through dietary intervention, followed by examination of aspects of

heart function in the absence of neuronal and hormonal input by using an isolated heart model.

Fatty acids and oxidation

Fatty acids occur in natural fats mainly as esters but also in the unesterified form as free fatty acids. They are normally single-chain derivatives containing an even number of carbon atoms. The chain may be saturated, containing no double bonds, or unsaturated, containing one or more double bonds.

The auto-oxidation of lipids exposed to oxygen is reported to be responsible for damage to tissues *in vivo*, where it may be a cause of cancer (Jiang, W.G. *et al.* 1998), inflammatory diseases (James, M.J. *et al.* 2000), atherosclerosis (Moore, K.J. & Freeman, M.W. 2006), aging (Praticò, D. 2002), and ischemia-reperfusion injury (Lucas, D.T. & Szweda, L.I. 1998). The damaging effects are deemed to be caused by free radicals produced during peroxide formation from fatty acids containing methylene-interrupted double bonds (Chapter Two, Figure 2.1) (Botham, K. & Mayes, P. 2009B). Therefore, the relative susceptibilities of different fatty acids to oxidation can be measured by calculating a peroxidation index (a measure of the susceptibility to peroxidation) (Hong, M.Y. *et al.* 2002; Kawai, Y. *et al.* 2006; Saito, M. & Kubo, K. 2003) or unsaturation index /double bond index (a measure of the density of carbon-carbon double bonds in the membrane) (Hong, M.Y. *et al.* 2002). The long chain, highly unsaturated (n-3) PUFA, DHA, found in fish oil, has six double bonds and five methylene carbons. It is more susceptible to peroxidation than other shorter, less unsaturated fatty acids such as the most predominant dietary (n-6) PUFA, linoleic acid, with two double

bonds and one methylene carbon, or the most predominant cellular (n-6) PUFA , arachidonic acid, with four double bonds and three methylene carbons (Rustan, A.C. 2005).

This high susceptibility to oxidation of (n-3) PUFA has long raised concerns about the potential adverse effects of fish oil supplementation (Demoz, A. *et al.* 1994; Leibovitz, B.E. *et al.* 1990; Nalbone, G. *et al.* 1989; Oostenbrug, G.S. *et al.* 1994; Suzukawa, M. *et al.* 1995). For example, dietary supplementation with (n-3) PUFA increases the oxidizibility of low density lipoprotein which could increase the risk of developing atherosclerosis (Suzukawa, M. *et al.* 1995). However, despite the high susceptibility of long chain (n-3) PUFA to oxidation, numerous studies have indicated that dietary (n-3) PUFA provide cardioprotective effects, as discussed in detail in Chapter Two (Marchioli, R. *et al.* 2001; Marchioli, R. *et al.* 2005; Mozaffarian, D. *et al.* 2005b).

Oxidative products are also implicated in ischemic preconditioning. One hypothesis regarding the mechanism of ischemic preconditioning is the membrane lipid hypothesis, which proposes that the brief stress caused by preconditioning would prevent ischemia-induced modification of membrane lipids (Jones, R.M. *et al.* 1992). Jones proposed that ischemic preconditioning may allow retention of a greater proportion of polyunsaturated fatty acids in membrane phospholipids, leading to preservation of membrane phospholipids and maintaining the membrane fluidity during ischemic insult, which may be at least partially responsible for the attenuation of ischemic reperfusion injury (Jones, R.M. *et al.* 1992).

On the basis of above information, it was the aim of experiments in this Chapter to test the hypotheses that:

- Fatty acid content of heart membranes will change in relation to the individual fatty acids and total polyunsaturated content of the experimental diets;
- Membrane phospholipid fatty acid composition will be influenced by ischemic preconditioning; and
- Peroxidization index in the heart membrane will be modified by inclusion of highly unsaturated fats in the diet.

4.2 METHODS

This section describes the diet treatment of rats, the steps of phospholipid analysis, which includes lipid extraction, phospholipid and triglyceride separation, preparation of fatty acid methyl esters and gas chromatography analysis.

Diet treatment

Groups of rats were separately treated for six weeks with diets that differed only in terms of types of fat in them, comprising 7% w/w beef tallow plus 3% w/w olive oil (SF diet), 7% w/w fish oil (NuMega high DHA tuna fish oil) plus 3%w/w olive oil (n-3 PUFA diet) or 5%w/w sunflower seed oil plus 5%w/w olive oil (n-6 PUFA

diet). The nutrient composition of the diets is detailed in Chapter Three. For baseline fatty acid measurements, fresh heart tissues (left ventricle) were obtained from rats immediately after they were sacrificed. For fatty acid measurements from hearts subjected to ischemic preconditioning, samples of non-ischemic regions of left ventricles were obtained on completion of the preconditioning and ischemia-reperfusion protocols outlined in Chapter Three. Tissue sampling and separation of ischemic region and non-ischemic region of the heart is described in Chapter Three. Tissues were stored in -80°C until use.

Phospholipid fatty acid analysis

The fatty acid composition of diet and heart tissue was determined by gas chromatography (Charnock, J.S. *et al.* 1992) following lipid extraction and conversion of fatty acids into methyl esters using a standard direct transesterification method (Lepage, G. & Roy, C.C. 1986). In this procedure, non volatile fatty acids are chemically converted to the corresponding volatile methyl esters. The resulting volatile mixture can be analysed by gas chromatography. In this study, a Shimadzu 17A gas chromotograph with flame ionization detection was used for the analysis. The data was captured on a computer by using Class VP software (version 7).

Lipid extraction

The total lipids were extracted from food or left ventricular tissue by a modification of the Folch and Sloane Stanley method (Folch, J. *et al.* 1957). The phospholipids

were isolated using solid phase extraction (Burdge, G.C. *et al.* 2000). The 50-200mg of frozen samples were weighed and homogenized in 4.5mL of chloroform: methanol mix (2:1, v/v) by hand using a glass tissue homogenizer. This ability of chloroform: methanol induces separation of the lipid protein mixture and even in some cases, denatures the protein. The homogenate was transferred to a screw cap tube and the homogenizers were washed with an additional 2mL chloroform: methanol (2:1) and was added to the same screw cap tube. Tubes were then rotated overnight at 4°C to separate the protein components from lipids in the sample. Next, in order to dissolve the nonlipid components of the membrane, 2mL of 1M H_2SO_4 was added to the samples and tubes were shaken vigorously for 30 seconds and then spun at 1000rpm for 10 minutes (Hettic Zentrifugen Rotina 46R). The top (aqueous) phase containing the acid and various non-lipid contaminants was removed and discarded while the bottom (solvent) phase containing the dissolved lipids was collected and transferred to plain glass screw top tubes (see Figure 4.3). Care was taken to avoid disturbing the protein disk between the two phases.

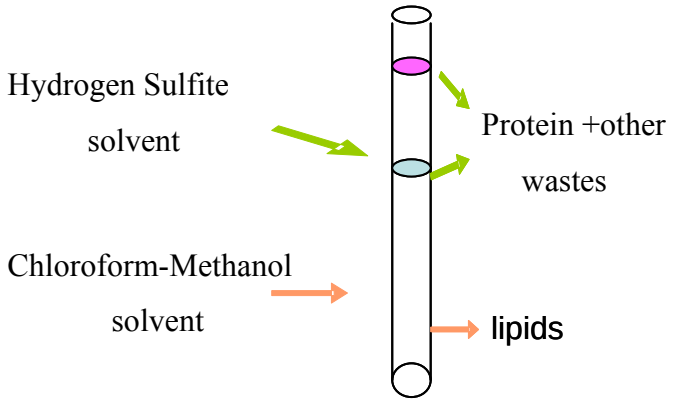


Figure 4.3 The separation of lipids

A further 2mL of 1M H_2SO_4 was added and the samples were again shaken vigorously, and spun at 1000rpm for 10 minutes. Again, the bottom (solvent) phase containing the lipids was collected and transferred to a clean tube. Then a small amount of (five taps) H_2SO_4 was added to the sample tube to bind any excess water and remove it from the sample for further purification of the sample. The sample was mixed and filtered through glass wool to remove the sodium hydrosulphite. Finally, the extracted lipids were collected in a tube. The sample was then dried by the addition of sodium dithionite and filtered through saline treated glass wool. The resulting filtrate represented the total lipid content of the ventricle containing phospholipids, cholesterol esters, triglyceride and other lipid like molecules.

Phospholipid and triglyceride separation

Phospholipids were separated by using solid phase extraction Sep-Pak® Silica cartridges (Waters, Sep-Pak). Total lipid samples were loaded onto the cartridges and neutral lipids were eluted by three washes of 30ml ethyl acetate, phospholipids were then eluted with three washes of 30ml of methanol. Phospholipid samples were then dried under nitrogen to about 1mL. After that, samples were transferred to screw cap tubes and stored overnight at – 20°C.

Preparation of fatty acid methyl esters and gas chromotograph analyses

Fatty acid methyl esters were prepared by direct transesterification of fatty acids in the phospholipids (the process of exchanging the alkoxy group of an ester compound with another alcohol). These reactions are catalyzed by the addition of an acid or base as described by Lepage (Lepage, G. & Roy, C.C. 1986). Extracted

phospholipids were dried under nitrogen and 2mL of methanol: toluene (4:1, v/v) was added to the phospholipids. While slowly vortexing the samples, 200uL of acetyl chloride was added to catalyse the methylation reaction. The tightly sealed samples were subjected to methanolysis by placing on the dry heat block at 100°C for 60 minutes. After transesterification with acetyl chloride, the pH of the solution is less than one and injection of this acidic benzene supernatant can break down the very thin stationary phase coating the capillary column. In order to avoid this problem, 5 ml of 6% K_2CO_3 was slowly added to the sample and vortexed to bring the pH back to neutral. To separate the toluene phase containing the fatty acid methyl esters, the samples underwent centrifugation for 5-10 minutes at 3000rpm and 4°C. The toluene layer was partially evaporated under nitrogen to concentrate the fatty acyl esters before being transferred to GC vials.

The fatty acid methyl esters (FAME) were then analysed by FID-GC (flame ionizing detector - gas chromatography) using a Shimadzu GC-17A with AOC-20i (automated sample injection system) with a 50 m (length) x 0.25 μm (wall coating) x 0.25 mm (internal diameter) CP-Select^{TM} CB for FAME capillary column (Varian, Middleburg, Netherlands. Catalogue number: CP7419). The program had a starting temperature of 150°C and ramp function of 20°C/minute for 2.3 minutes followed by a ramp function of 3.5°C/minute for 9 minutes then 0.5°C/minute for 14 minutes and after that 3.5°C/minute for 1.7 minutes. The temperature was then held for six minutes, giving a total run time of 33 minutes. The final temperature of the detector and injector was 260°C. Ultra high purity hydrogen was used as the carrier gas and the column flow rate was 0.8ml/minute. Peak identification and quantification was

carried out using Class VP software (version 7) by Shimadzu. Peaks were identified by comparison with known retention times. Retention times were defined as the elapsed time between the time of injection of a sample and the time of elution of a particular. Each peak represents a different fatty acid identified by matching its retention time to peaks of known fatty acid methyl ester (FAME) standards. For each sample run of up to 25 samples, two FAME reference standards were used (NuChek Prep, Elysian, MN, USA, catalogue number: GLC 673B and Sigma-Aldrich Qualitative F.A.M.E. Mix, C4-C24, Catalogue number: 18919-1AMP). Fatty acids were quantified by calculation of peak area, and are expressed as a percentage of the total fatty acids measured. Fatty acid standards were run along with each batch of all samples. Sample data traces from the fatty acid standard showing the retention times and the percentage contribution of each fatty acid to the total and identification of each fatty acid are shown in Figure 4.4A, B, and C.

Raw data trace

In the heart samples, individual fatty acids were identified by comparing the retention times for each peak against a standard (Figure 4.4A). In the sample from a (n-6) PUFA heart (Figure 4.4C), linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) present peaks of larger area than DHA (22:6n-3) or EPA (20:5n-3). In contrast, in a (n-3) PUFA heart sample (Figure 4.4B), DHA (22:6n-3) presents a peak of greater area than linoleic acid (18:2n-6) or arachidonic acid (20:4n-6). This indicates the greater incorporation of (n-6) PUFA in the heart of a (n-6) PUFA -fed rat and greater incorporation of (n-3) PUFA in the heart of a rat fed (n-3) PUFA. Within the (n-3) PUFA, DHA presents a peak of larger area than does EPA in both

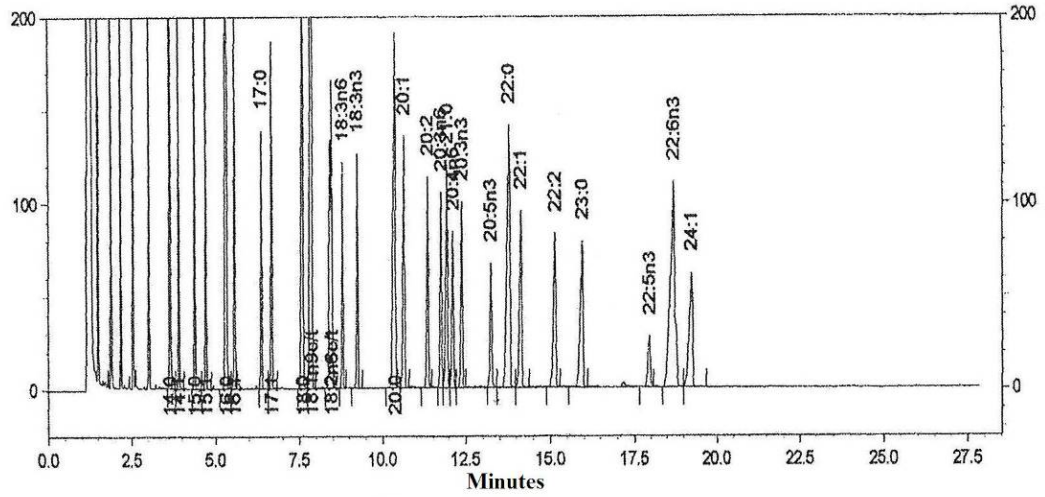
dietary group samples. A SF heart sample trace (not shown) would appear very similar to the (n-6) PUFA heart sample (see table 4.2)

Fat composition of the diet

Diets fat to the rats were fully fabricated in our laboratory to be iso-energetic, containing 10% fat (22% en) enriched with either predominantly saturated fat, (n-6) PUFA or (n-3) PUFA. Fatty acid composition of rat food is shown in Table 4.1.

Statistical analyses

Statistics were conducted using SPSS 15.0 version for Windows. For tissue fatty acid composition analysis, comparison was made between dietary groups and preconditioned groups with two-way analysis of variance, with Tukey's post hoc test for comparison of individual means. For diet fatty acid composition analysis, one way ANOVA was used to determine any differences between diet groups, using post-hoc TUKEY test. $P<0.05$ was accepted as statistically significant. Data are represented as mean ±SEM.

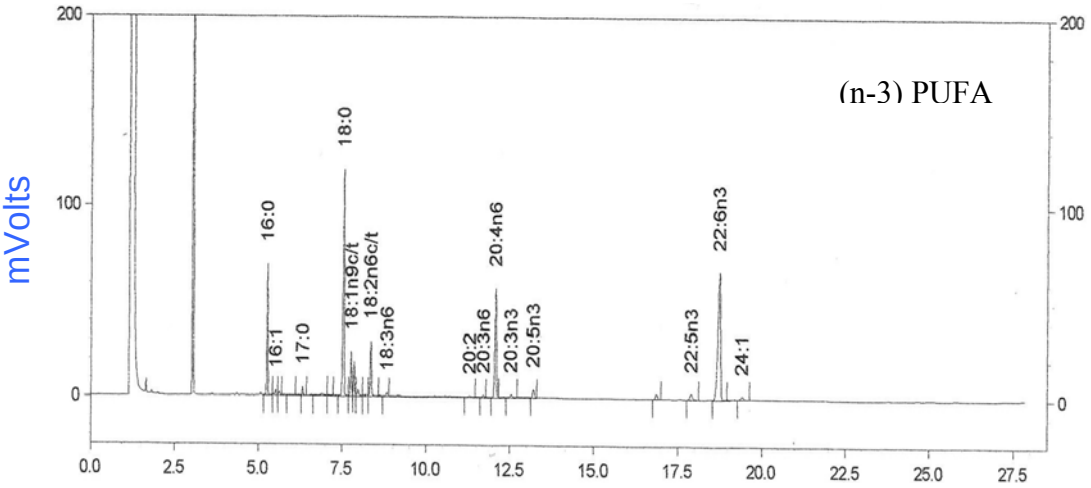


FID Results

Pk #	Retention Time	Area	Area %	Peak name
1	3.641	839980	5.411	14:0
2	3.900	408978	2.635	14:1
3	4.391	431442	2.779	15:0
4	4.716	426268	2.746	15:1
5	5.333	1314032	8.465	16:0
6	5.566	403411	2.599	16:1
7	6.358	328568	2.117	17:0
8	6.658	435586	2.806	17:1
9	7.600	892064	5.746	18:0
10	7.866	1326204	8.543	18:1n9c/t
11	8.449	796545	5.131	18:2n6c/t
12	8.783	301594	1.943	18:3n6
13	9.241	333801	2.150	18:3n3
14	10.383	871113	5.611	20:0
15	10.641	449488	2.895	20:1
16	11.349	386565	2.490	20:2
17	11.766	343649	2.214	20:3n6
18	11.949	456424	2.940	21:0
19	12.124	287381	1.851	20:4n6
20	12.391	343987	2.216	20:3n3
21	13.241	252053	1.624	20:5n3
22	13.799	912588	5.879	22:0
23	14.149	440059	2.835	22:1
24	15.141	395539	2.548	22:2
25	15.949	454477	2.928	23:0
26	17.957	154457	0.995	22:5n3
27	18.715	1094907	7.053	22:6n3
28	19.240	442636	2.851	24:1
		15523796	**100.000**	

Figure 4.4A Raw data and results from a fatty acid standard (Sigma-Aldrich Qualitative F.A.M.E. Mix, C4-C24, Catalogue number: 18919-1AMP) showing retention times of each individual fatty acid and its relative area.

B



C

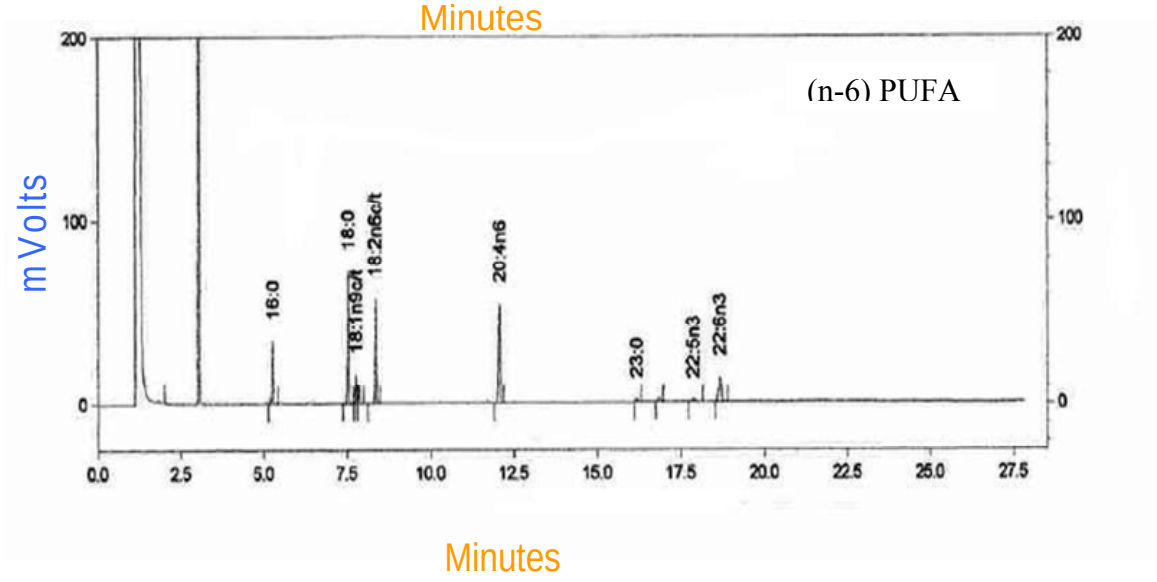


Figure 4.4B,C Sample raw data trace from (n-3) PUFA(B) and (n-6) PUFA(C) hearts showing retention times of each individual fatty acid and its relative area.

Table 4.1 Fat and Fatty acid composition of rat diets

	SF	(n-6) PUFA	(n-3) PUFA
		g/kg of diet	
Fish oil			70
Sunflower oil		50	
Beef tallow	70		
Olive oil	30	50	30
	% of total fatty acids		
12:0	0.6	n.d	n.d
14:0	2.6	n.d	2.1
16:0	20.6	8.1	17.1
16:1	3.2	0.4	3.4
18:0	14.1	3.6	4.5
18:1 (Oleic acid)	47.9	50.0	33.5
18:2n-6 (Linoleic acid)	4.7	36.0	3.5
18:3n-3 (Alpha linolenic acid)	0.6	0.5	0.6
20:4n-6 (Arachidonic acid)	0.1	n.d	1.3
20:5n-3 (Eicosapentaenoic acid)	n.d	n.d	4.9
22:5n-3 (Docosapentaenoic acid)	n.d	n.d	0.8
22:6n-3 (Docosahexaenoic acid)	n.d	n.d	20.2
Total saturated fatty acids	38.1	12.6	25.5
Total monounsaturated fatty acids	51.4	50.6	39.2
Total PUFA	5.4	36.6	31.4
Total (n-6) PUFA	4.8	36.2	5.0
Total (n-3) PUFA	0.6	0.5	26.4
P:S ratio	0.25	3.18	1.14
(n-6):(n-3) PUFA	8.3	74	0.19

P:S ratio = polyunsaturated to saturated fatty acid ratio
n.d= not detected

4.3 RESULTS

Influence of dietary supplements on phospholipid fatty acid composition of isolated rat heart

Dietary supplementation with (n-3) PUFA for six weeks resulted in changes to the phospholipid fatty acid composition of myocardial membranes compared to the other diets (Table 4.2). The docosahexaenoic acid (DHA: 22:6n-3) in (n-3) PUFA hearts was significantly higher than DHA in (n-6) PUFA and SF hearts ($p<0.05$). There was a significant difference in DHA between (n-6) PUFA (10.02±0.2) hearts and SF (12.2±0.04) hearts (Figure 4.5a).

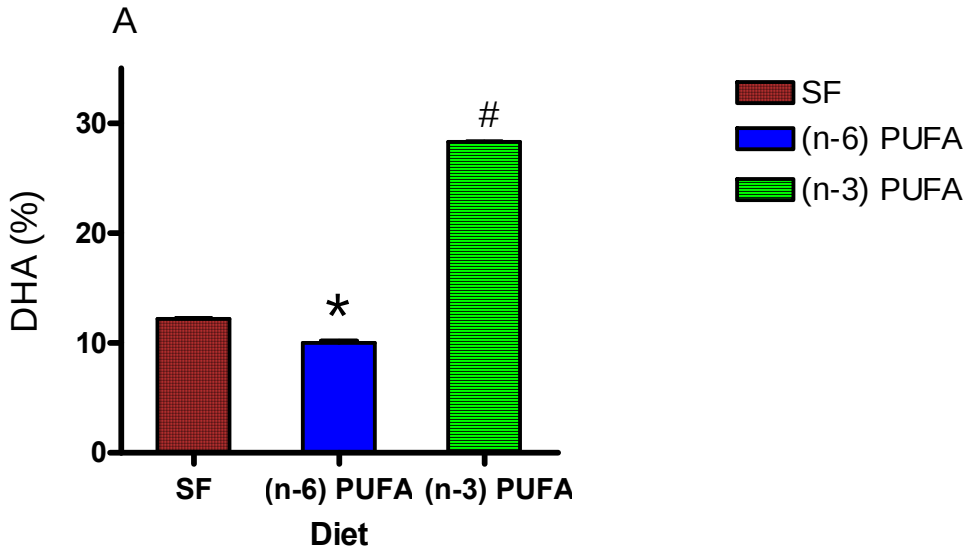


Figure 4.5A The percentage of DHA in heart membrane of rats that consumed the SF, (n-6) PUFA or (n-3) PUFA diets for 6 wk. Crossed (Red) bar shows SF heart, Filled (blue) bar shows (n-6) PUFA heart, striped (green) bar shows (n-3) PUFA heart. Values are means ± SEM, n=6-9. # Different to SF and (n-6) PUFA dietary group, $P < 0.05$. * Different to SF dietary group, $P < 0.05$.

The (n-3) PUFA hearts contained significantly lower concentrations of oleic acid (18:1n-9) (Table 4.2) linoleic acid (18:2n-6) (Figure 4.5B) and arachidonic acids (20:4n-6) (Figure 4.5C) compared to (n-6) PUFA and SF hearts. There was a small statistical difference in linoleic acid (18:2n-6) but no difference in arachidonic acid concentration between (n-6) PUFA and SF hearts.

The total (n-3) PUFA concentration was higher in (n-3) PUFA hearts compared to (n-6) PUFA and SF hearts (Figure 4.5D) and total (n-6) PUFA concentration was lower in (n-3) PUFA hearts compared to (n-6) PUFA and SF hearts (Table 4.2). The total PUFA was lower in (n-6) PUFA hearts than SF hearts (Table 4.2).

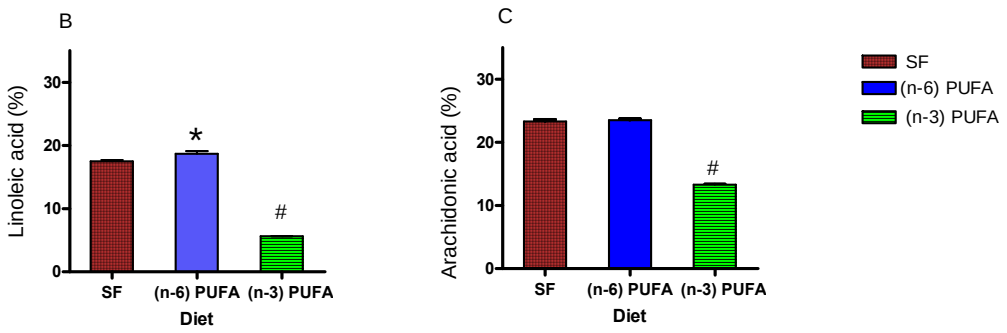


Figure 4.5B,C The percentage of B: Linoleic acid C: arachidonic acid in heart membrane of rats that consumed the SF, (n-6) PUFA or (n-3) PUFA diets for 6 wk. Crossed (Red) bar shows SF heart, Filled (blue) bar shows (n-6) PUFA heart, striped (green) bar shows (n-3) PUFA heart. Values are means ± SEM, n=6-9. # Different to SF and (n-6) PUFA dietary group, $P < 0.05$. * Different to SF dietary group, $P < 0.05$.

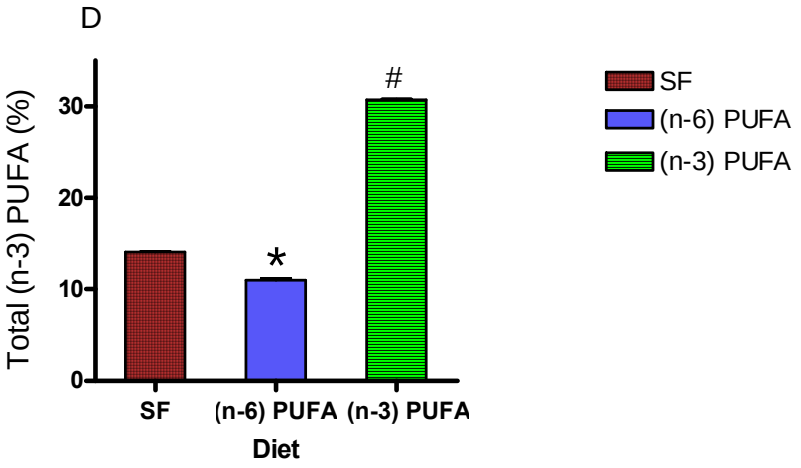


Figure 4.5D The percentage of total (n-3) PUFA in heart membrane of rats that consumed the SF, (n-6) PUFA or (n-3) PUFA diets for 6 wk. Crossed(Red) bar shows SF heart, Filled (blue) bar shows (n-6) PUFA heart, striped (green) bar shows (n-3) PUFA heart. Values are means ± SEM, n=6-9. # Different to SF and (n-6) PUFA dietary group, $P < 0.05$. * Different to SF dietary group, $P < 0.05$.

Unsaturation index (Figure 4.5E) and peroxidization index (Figure 4.5F) were significantly higher in (n-3) PUFA than in (n-6) PUFA and SF hearts, which were not significantly different. Full details of heart phospholipid fatty acid composition are shown in Table 4.2.

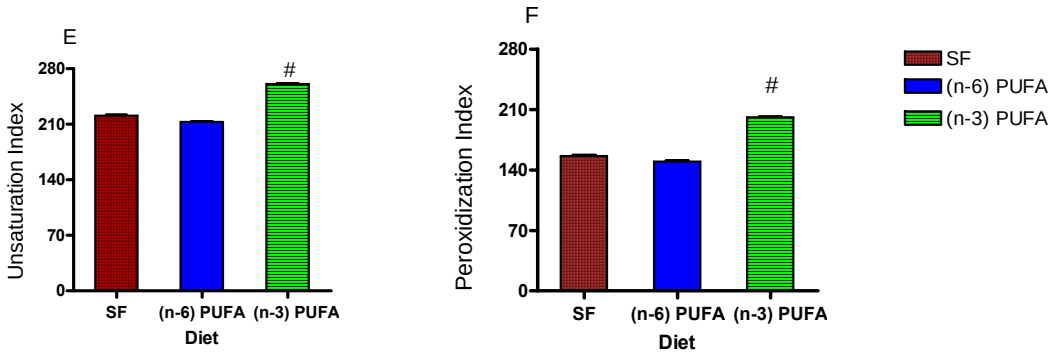


Figure 4.5E,F The E: unsaturation index, F: peroxidization index of different fatty in heart membrane of rats that consumed the SF, (n-6) PUFA or (n-3) PUFA diets for 6 wk. Crossed(Red) bar shows SF heart, Filled (blue) bar shows (n-6) PUFA heart, striped (green) bar shows (n-3) PUFA heart. Values are means ± SEM, n=6-9. # Different to SF and (n-6) PUFA dietary group, $P < 0.05$.

Influence of ischemic preconditioning on phospholipid fatty acid composition of isolated rat heart membrane after diet treatment

Hearts that were subjected to ischemic preconditioning as described in Chapter Three followed by 30 minutes regional ischemia and 120 minutes reperfusion exhibited dietary effects that were no different from those observed in the myocardial membrane phospholipid fatty acids of fresh hearts (Table 4.3).

4.4 DISCUSSION

The present study was conducted to evaluate the changes in heart membrane fatty acid composition produced by a six-week dietary treatment of rats and whether ischemic preconditioning causes any changes in heart membrane fatty acid composition in addition to diet.

While heart membrane fatty acids changed after feeding different diets, they did not exactly replicate what is in the diet. The content of the saturated fatty acids, for example, stearic acid, was high in the saturated fat diet, and low in (n-6) PUFA and (n-3) PUFA diets, yet the amount of stearic acid in the heart membranes were almost the same. Regardless of the low DHA content in the diet, the DHA in SF membrane was higher than the DHA in (n-6) PUFA membrane. This indicates that the animal body has the capacity to incorporate these fatty acids into the membrane in a controlled fashion to maintain membrane structure and function (Botham, K. & Mayes, P. 2009A).

Table 4.2 Fatty acid composition of rat heart membranes as % of total fatty acids: Effect of dietary supplements

Fatty acids	SF	(n-6) PUFA	(n-3) PUFA
16:00 Palmitic acid	^{b}9.6 ± 0.1	^{ab}10.1 ± 0.3	^{a}10.7 ± 0.1
18:00 Stearic acid	^{a}24.2 ± 0.2	^{ab}23.6 ± 0.1	^{b}22.9 ± 0.2
18:1 n-7 Vaccenic acid	3.6 ± 0.3	3.5 ± 0.1	3.4 ± 0.8
18:1 n-9 Oleic acid	^{a}9.5 ± 0.1	^{b}5.4 ± 0.1	4.3 ± 0.3
18:2 n-6 Linoleic acid	^{b}17.5 ± 0.2	^{a}18.7 ± 0.4	5.6 ± 0.0
20:4 n-6 Arachidonic acid	^{a}23.3 ± 0.3	^{a}23.5 ± 0.2	13.3 ± 0.1
20:5 n-3 Eicosapentaenoic acid	n.d	n.d	1.3 ± 0.0
22:5 n-6 Docosapentaenoic acid	n.d	^{a}1.5 ± 0.1	1.0 ± 0.0
22:5 n-3 Docosapentaenoic acid	^{a}1.9 ± 0.1	1.0 ± 0.0	1.1 ± 0.0
22:6 n-3 Docosahexaenoic acid	^{b}12.2 ± 0.1	10.0 ± 0.2	^{a}28.3 ± 0.0
Total saturated FA	33.8 ± 0.1	33.7 ± 0.8	33.7 ± 0.4
Total PUFA	54.9 ± 4.5	54.7 ± 4.5	50.7 ± 4.4
Total (n-6) PUFA	^{b}40.8 ± 0.2	^{a}43.8 ± 0.6	20.0 ± 0.1
Total (n-3) PUFA	^{b}14.1 ± 0.1	11.0 ± 0.2	^{a}30.7 ± 0.0
Unsaturation Index	220.4 ± 1.2	212.9 ± 0.5	260.5^{a} ± 1.2
Peroxidization Index	156.4 ± 1.2	149.5 ± 1.6	^{a}201.1 ± 0.1

Values sharing a common letter superscript are not significantly different by row.
Unsaturation index (UI) was calculated according to the formula: UI = 1 x (% monoenoics) + 2 x (% dienoics) + 3 x (% trienoics) + 4 x (% tetraenoics) + 5 x (% pentaenoics) + 6 x (% hexaenoics) or sum (fatty acid percent) × (number of double bonds) (Lee, J. *et al.* 1999; Llanillo, M. *et al.* 1995).
Peroxidization index was calculated from following equation,
Peroxidization index = (% dienoic acid × 1) + (% trienoic acid × 2) + (% tetraenoic acid × 3) + (% pentaenoic acid × 4) + (% hexaenoic acid × 5). (Kawai, Y. *et al.* 2006; Saito, M. & Kubo, K. 2003) (Hong, M.Y. *et al.* 2002).n.d: not detected n=6

Table 4.3 Fatty acid composition of membranes from hearts subjected to ischemic preconditioning expressed as % total fatty acids

Fatty acids	SF	(n-6) PUFA	(n-3) PUFA
16:00 Palmitic acid	^{b}9.7 ± 0.0	^{ab}10.2 ± 0.2	^{a}10.8 ± 0.1
18:00 Stearic acid	^{a}23.7 ± 0.1	^{ab}23.8 ± 0.2	^{b}22.4 ± 0.3
18:1 n-7 Vaccenic acid	3.6 ± 0.2	3.5 ± 0.1	3.4 ± 0.8
18:1 n-9 Oleic acid	^{a}9.4 ± 0.1	^{b}5.3 ± 0.2	4.3 ± 0.4
18:2 n-6 Linoleic acid	^{b}17.8 ± 0.3	^{a}19.1 ± 0.2	5.5 ± 0.0
20:4 n-6 Arachidonic acid	^{a}22.7 ± 0.2	^{a}23.7 ± 0.2	13.2 ± 0.2
20:5n-3 Eicosapentaenoic acid	n.d	n.d	1.5 ± 0.0
22:5 n-6 Docosapentaenoic acid	n.d	^{a}1.4 ± 0.2	1.0 ± 0.0
22:5 n-3 Docosapentaenoic acid	^{a}1.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.0
22:6 n-3 Docosahexaenoic acid	^{b}12.5 ± 0.0	10.1 ± 0.3	^{a}28.4 ± 0.0
Total saturated FA	33.4 ± 0.1	34.0 ± 0.9	33.2 ± 0.3
Total PUFA	54.2 ± 4.6	55.4 ± 4.5	49.3 ± 4.5
Total (n-6) PUFA	^{b}40.5 ± 0.3	^{a}44.2 ± 0.6	19.8 ± 0.2
Total (n-3) PUFA	^{b}14.4 ± 0.0	11.1 ± 0.3	^{a}31.0 ± 0.1
Unsaturation Index	220.5 ± 1.3	211.2 ± 0.6	261.0^{a} ± 1.2
Peroxidization Index	156.1 ± 1.3	150.6 ± 1.7	^{a}202.6 ± 0.8

Values sharing a common letter superscript are not significantly different by row. Unsaturation index (UI) was calculated according to the formula: UI = 1 x (% monoenoics) + 2 x (% dienoics) + 3 x (% trienoics) + 4 x (% tetraenoics) + 5 x (% pentaenoics) + 6 x (% hexaenoics) or sum (fatty acid percent) × (number of double bonds) (Lee, J. *et al.* 1999; Llanillo, M. *et al.* 1995). Peroxidization index was calculated from following equation. Peroxidization index = (% dienoic acid × 1) + (% trienoic acid × 2) + (% tetraenoic acid × 3) + (% pentaenoic acid × 4) + (% hexaenoic acid × 5) (Hong, M.Y. *et al.* 2002; Kawai, Y. *et al.* 2006; Saito, M. & Kubo, K. 2003). n.d: not detected n=6

Furthermore, regardless of high saturated fat content and high (n-6) PUFA content in different diets, the percentage of individual fatty acids in membrane of SF and (n-6) PUFA fed rats was similar.

These findings are in contrast to studies by Charnock and Mclennan where they found high membrane (n-6) PUFA concentration in rat hearts fed with high linoleic acid sunflower seed oil compared to saturated fat (Charnock, J.S. *et al.* 1983; McLennan, P.L. *et al.* 1993). The discrepancy to the present study may be largely due to the fact that Charnock& Mclennan used 12% sunflower seed oil or 12% sheep perirenal fat as the sole source of added fat, whereas the present study used only 5% sunflower seed oil or 7% animal fat blended with olive oil. The 5% sunflower seed oil in present study may not have been enough to cause any higher incorporations of (n-6) PUFA in the membrane. Alternatively the high saturated fat intake could have created an essential fatty acid deficiency and the very low PUFA affected the membrane saturation. The levels of saturated fat, linoleic acid and arachidonic acid in present study were similar to the study conducted by Owen *et al* (Owen, A.J. *et al.* 2004), in which they used an olive oil diet. Either pure olive oil itself or the combination of olive oil plus saturated fat and olive oil plus sunflower seed oil seem to produce similar changes in the heart membrane (Owen, A.J. *et al.* 2004). It appears that only extremes of saturated or (n-6) PUFA in the diet produce major changes in the membrane. The failure of (n-6) PUFA to greatly change membrane unless extremely high intakes are delivered suggests that the membrane incorporates a base concentration irrespective of dietary supply once essential fatty acid deficiency has been overcome.

In present study, the (n-6) PUFA diet provided a high linoleic acid concentration and both the SF diet and (n-3) PUFA diet provided less linoleic acid than the American Institute of Nutrition recommendations for growing rats (Reeves, P.G. 1997), yet the linoleic acid composition in heart membrane between SF diet and (n-

6) PUFA diet were very similar. The animal growth data also indicated that the low linoleic acid concentration of the SF diet did not effect animal growth (Chapter Three). The rats used in this study were mature (9 weeks old) and 340g at commencement, yet managed to gain in excess of 3 g/day over the duration of the study (data shown in Chapter Three). The lower requirement for linoleic acid in the diet is supported by several studies (Cunnane, S.C. & Anderson, M.J. 1997; Holman, R.T. 1971). The current finding suggest that the low concentration of linoleic acid in saturated fat diet and (n-3) PUFA diet had no impact on rat growth and the requirements for linoleic acid are substantially less than the 2% energy normally recommended. Specifically, Bourre *et al.* demonstrated that linoleic fatty acid provided at 0.4% of energy intake supports normal rat growth and reproduction as well as maintaining normal arachidonic acid concentrations in all organs (Bourre, J.M. *et al.* 1990). Thus, 2% of energy recommended for dietary linoleic acid (Reeves, P.G. 1997) may well exceed the minimum requirement for adequate growth and tissue (n-6) PUFA accumulation in free-feeding animals (Bourre, J.M. *et al.* 1990). In the present study, linoleic acid provided approximately 1% of energy intake (total percent energy as fat) in SF diet and (n-3) PUFA diets, which was above the level of 0.4% recommended by Bourre and associates (Bourre, J.M. *et al.* 1990). Whereas the (n-6) PUFA diet provided almost 8%en as linoleic acid and there were no apparent health or growth differences over six weeks.

In contrast to the minor differences in the membrane between the SF and (n-6) PUFA diets, feeding rats with fish oil caused major alterations in the membrane. The main influence was incorporation of DHA into heart phospholipid membrane when the diet had been supplemented with fish oil. Although this study used a high

DHA fish oil, other studies show that even after using high EPA fish oils, it is DHA that increases in the heart membranes, with very little change in EPA (Hock, C.E. *et al.* 1990; McLennan, P.L. *et al.* 1996). A diet containing 12% of the fat as DHA and 28% as EPA produces rat myocardial membranes containing less than 5% EPA and more than 20% DHA. In turn, a fish oil diet containing 28% DHA and 12% EPA can produce a myocardial EPA concentration of less than 1% and a DHA concentration of more than 30%, whereas consumption of a fish oil–free diet will result in undetectable EPA concentration and less than 10% DHA (Charnock, J.S. *et al.* 1992). In summary, regardless of the relative intake of EPA and DHA, it is DHA that increases in the heart membrane.

It is well established that DHA is the main (n-3) PUFA found in myocardial phospholipids in most species including rats (Hock, C.E. *et al.* 1990; Owen, A.J. *et al.* 2004), monkeys (Charnock, J.S. *et al.* 1989; Charnock, J.S. *et al.* 1992) pigs (Nair, S.S. *et al.* 2000) and humans (Gudbjarnason, S. & Hallgrimsson, J. 1976; Harris, W.S. *et al.* 2004; Rocquelin, G. *et al.* 1985; Rocquelin, G. *et al.* 1989), with EPA generally undetectable in the absence of supplementation by fish oil feeding.

In other tissues such as animal liver, kidney (Charnock, J.S. *et al.* 1992), animal skeletal muscle (Charnock, J.S. *et al.* 1989; Charnock, J.S. *et al.* 1992; Owen, A.J. *et al.* 2004), human skeletal muscle (Baur, L.A. *et al.* 2000; Helge, J.W. *et al.* 2001), human plasma (Conquer, J.A. *et al.* 2002; Dewailly, E. *et al.* 2002), DHA is also the predominant (n-3) PUFA, irrespective of the background diet. Although few studies reported the relative differences between EPA and DHA concentrations

in erythrocyte membranes of either humans and rats, available data suggested that EPA is more prominent in the fatty acid profiles of red blood cells compared with heart (Sexton, P.T. *et al.* 1995) and the incorporation of EPA and DHA into erythrocyte membranes was at the expense of oleic and linoleic acid rather than the long chain (n-6) PUFA arachidonic acid (Sexton, P.T. *et al.* 1995). Overall, myocardium seems to have a special ability to incorporate DHA rather than EPA.

In present study, this increased incorporation of DHA into the heart membrane was associated with decreased concentrations of linoleic and arachidonic acid. These changes in fatty acid composition are in agreement with the reports of other researchers who have shown displacement of arachidonic acid in animals (Al Makdessi, S. *et al.* 1994; Charnock, J.S. *et al.* 1985; Hartog, J.M. *et al.* 1987; McLennan, P.L. 1993; Swanson, J.E. & Kinsella, J.E. 1986). This displacement of arachidonic acid (20:4n-6 PUFA) by (n-3) PUFA, particularly DHA (22:6n-3 PUFA) also has been demonstrated in human hearts, where patients were supplemented with (n-3) PUFA oil prior to cardiac surgery during which a biopsy was taken (Metcalf, R.G. *et al.* 2007).

The metabolic pathway from linoleic acid (18:2 *n-6* PUFA) to arachidonic acid (20:4 n-6 PUFA) involves desaturation at the Δ5 and Δ6 positions in the carbon backbone and an intermediate 2-carbon chain elongation step (Chapter Two, Figure 2.2). Studies suggest that, DHA is preferentially incorporated into membrane phospholipids, not only by displacing arachidonic acid, but also inhibiting the enzyme Δ6 desaturase (Raz, A. *et al.* 1997). As a consequence, DHA reduces the

amount of linoleic acid that is converted to arachidonic acid (Brenner, R.R. 1971, 1981). Inhibition of Δ5 desaturase would also further limit arachidonic acid incorporation by prohibiting the final step in the conversion of dihomo-gamma-linolenic acid (20:3 n-6PUFA) to arachidonic acid (20:4 n-6 PUFA). The present study actually showed that the concentration of linoleic acid decreased due to displacement by DHA in the membrane. However, the effects of DHA on desaturase activity cannot be determined from the present data.

Although the inhibition of synthesis or displacement of arachidonic acid by DHA is likely to diminish the synthesis of powerful, arachidonic acid –derived mediators of inflammation, such as thromboxane, prostaglandins and leukotrienes (Calder, P.C. 2006), the high unsaturation index and peroxidation index of DHA in cardiac phospholipids has caused speculation about potential adverse consequences of DHA on heart function (Song, J.H. & Miyazawa, T. 2001). The presence of this highly unsaturated fatty acid makes heart membranes more susceptible to lipid peroxidation (Nalbone, G. *et al.* 1988). In line with an other study (Al Makdessi, S. *et al.* 1994), the present study found a marked increase in unsaturation index and peroxidation index in (n-3) PUFA hearts compared to (n-6) PUFA and SF hearts. This suggests that (n-3) PUFA hearts are more susceptible to peroxidation. Despite The (n-6) PUFA diet presenting the highest concentration of total polyunsaturated fatty acids of any diet, the (n-6) PUFA hearts failed to show any increase in unsaturation index and peroxidization index compared to SF diet. This suggests some value in investigating the role of membrane lipid oxidation in heart function. The consequences of these changes in the membrane peroxidization index are tested by measuring ischemic damage (reported in Chapter Five) and oxidative

products and antioxidant status both at rest and after ischemia and reperfusion (reported in Chapter Six).

Ischemic-preconditioning did not modify the membrane fatty acid composition within any dietary groups (Table 4.3). This is in agreement with a study conducted by al Makdessi and colleagues (Al Makdessi, S. *et al.* 1995), which found no changes in the fatty acids of preconditioned hearts. In contrast, a lengthy period of regional ischemia followed by reperfusion in pig heart decreased oleic, linoleic, and arachidonic fatty acids in heart membrane phospholipids (Jones, R.M. *et al.* 1992). Overall, this present study has found no measurable changes in fatty acid composition of myocardial phospholipids 150 minutes after ischemic preconditioning and demonstrated that the alteration of polyunsaturated phospholipid fatty acid concentrations is not a pre-requisite for the effects of ischemic preconditioning. This also suggests that, in general, the metabolic integrity of the membranes was maintained throughout three hours perfusion, including ischemia and reperfusion.

In summary, six weeks dietary treatment of rats with fish oil in present study caused substantial alterations in myocardial phospholipid fatty acid composition. Fish oil feeding promoted incorporation of (n-3) PUFA, DHA, which has been associated with beneficial effects on arrhythmia and oxygen consumption in the heart but at the same time increased the peroxidation index suggesting increased susceptibility of the membrane to oxidative damage, which might be expected to enhance ischemic damage. Following chapters will evaluate the susceptibility to oxidative changes together with functional effect and infarct size after ischemia and reperfusion.

CHAPTER 5

THE PRECONDITIONING EFFECT OF DIETARY (N-3) LONG CHAIN POLYUNSATURATED FATTY ACIDS IN ISOLATED RAT HEART

5.1 INTRODUCTION

Regular intake of (n-3) PUFA through fish or fish oil is associated with reduced mortality from ischemic heart disease in both epidemiological studies and clinical trials (Albert, C.M. *et al.* 2002; Burr, M.L. *et al.* 1989a; Valagussa, F. *et al.* 1999). Clinical intervention has shown (n-3) PUFA intake to be associated with prevention of fatal cardiac arrhythmias that can occur early in an ischemic episode or during reperfusion, even though the incidence of ischemic events may not be affected (Marchioli *et al.* 2002). The effects of (n-3) PUFA on ischemia have also been studied extensively in animals. Experimental evidence suggests that regular consumption of (n-3) PUFA is particularly effective in protecting against the damaging effects of myocardial ischemia. For example, feeding animals (n-3) PUFA, enhances early post-ischemic recovery of heart function (Pepe, S. & McLennan, P.L. 2002; Yang, B. *et al.* 1993), prevents arrhythmias in association with ischemia and reperfusion (Matthan, N.R. *et al.* 2005), reduces myocardial oxygen consumption without diminishing work output, and reduces the production

of early markers of ischemic damage that are regarded as predictive of infarct size (Pepe, S. & McLennan, P.L. 2002; Pepe, S. 2007). Above studies, illustrate both clinical and experimental data that together provide evidence of protection against the morbidity and mortality of ischemic heart disease.

Cardioprotection against damaging effects of ischemia may also be provided through the phenomenon of ischemic preconditioning, wherein brief periods of acute myocardial ischemia provide some protection for the heart against the damaging effects of subsequent prolonged episodes of ischemia. Similar to the cardioprotective effects of fish oil, ischemic preconditioning is also proven to provide cardioprotection, as illustrated by: lower heart muscle oxygen demand; improvement in the recovery of post-ischemic heart function; reduction in the incidence of ischemia-reperfusion induced arrhythmia; and reduction of infarct size (Cohen, M.V. *et al.* 1991; Hagar, J.M. *et al.* 1991; Shiki, K. & Hearse, D.J. 1987). This cardioprotective action of ischemic preconditioning, which was first demonstrated in dogs (Murry, C.E. *et al.* 1986), has subsequently been confirmed using rats, rabbits and other animal models. Its demonstration in humans as well (Heusch, G. 2001; Tomai, F. *et al.* 1999), has invigorated a search to establish a viable means of utilising preconditioning therapeutically (Yellon, D.M. & Downey, J.M. 2003).

Ischemia generates numerous metabolites, autacoids and cell signaling molecules shown to act as triggers of preconditioning (Cohen, M.V. *et al.* 2000), and many are under investigation for the potential development of therapeutic approaches to

preconditioning, so called pharmacological preconditioning (Kloner, R.A. & Rezkalla, S.H. 2006; Szekeres, L. 2005). However, despite advances in identifying pharmacological approaches to mimic ischemic preconditioning, the intra-cellular processes targeted are common to many cell types (Otani, H. 2008). Therefore, they tend to lack cardiac specificity and have inherent potential for side effects, which together with poor prospects of predicting ischemic episodes, brings into question the feasibility of such an approach. These problems largely limit the potential use of pharmacological preconditioning to specific situations such as during coronary artery bypass graft (Kloner, R.A. 2006; Kloner, R.A. & Rezkalla, S.H. 2006).

In the previous chapter of this thesis, fatty acid analysis confirmed that feeding a fish oil diet changes the composition of myocardial membrane by incorporating an increased concentration of DHA. The clinical and epidemiological evidence for cardioprotective effects of fish oil, together with specific experimental evidence of preconditioning–like effects on heart function suggests that the presence of (n-3) PUFA in the heart provides a form of protection that might be seen as a nutritional equivalent of ischemic preconditioning. A dietary approach with a safe and effective nutritional component could overcome the need to predict the onset of ischemic episodes, which currently constrains the potential effectiveness of pharmacological therapies.

This present study aimed to evaluate the efficacy of dietary fish oil in providing cardioprotection by evaluating it under the same conditions as those demonstrating ischemic preconditioning and evaluating their potential synergy. It specifically

tested the hypothesis that (n-3) PUFA would provide sustained recovery of myocardial function following ischemia-reperfusion. This is preparatory to investigating the cell signalling mechanisms underpinning (n-3) PUFA-mediated cardioprotection.

5.2 METHODS

Rats were fed SF diet, (n-6) PUFA diet and (n-3) PUFA diet for six weeks as described in Chapter Three. At the end of six weeks, the rats were anaesthetized and their excised heart was attached to a Langendorff perfusion apparatus. The Langendorff coronary perfusion set up and intraventricular pressure measurement are described in Chapter Three. The heart was perfused in the Langendorff mode under constant aortic pressure of 75mmHg with intraventricular end diastolic pressure initially adjusted to 6-8mmHg.

Coronary flow was measured by timed collection of the coronary effluent. Heart rate, maximum rate of pressure development, maximum rate of relaxation and left ventricular developed pressure were determined by analysing pressure tracings using Lab View for Windows. The electrocardiogram (ECG) was recorded continuously with two platinum alloy needle electrodes (Grass instruments, Quincy, MA) placed at the surface of ventricular apex and aortic stump. The experiment described here involved the induction of regional ischemia through ligation of the left anterior descending coronary artery as described in Chapter Three. Heart

function was measured including the assessment of cardiac arrhythmias in ischemia and reperfusion.

Amongst the arrhythmias, ventricular tachycardia was defined as four or more consecutive beats of similar morphology with no preceding P wave and with a basic cycle length at least 20% less than that of prevailing complexes. Ventricular fibrillation was defined as chaotic morphology of the repetitive complexes for at least four cycles accompanied by a precipitous drop in developed pressure. Arrhythmias were additionally assessed by counting the number of ventricular premature beats and the incidence and total duration of all episodes of ventricular tachycardia and ventricular fibrillation. Global severity of arrhythmias was assessed using scores for ischemia and reperfusion (McLennan, P.L. *et al.* 1988). The score awarded points on a hierarchical scale of 0-9. A score of 0-5 represents increasing severity of reverting arrhythmias. A score of 6-9 represents the occurrence of non-reverting ventricular fibrillation of progressively earlier onset. Hearts were cardioverted by manual stimulus after 5-minute continuous arrhythmia.

Ischemic protocol

Control: Control experiments represent those in which hearts from rats that were fed any one of the diets were subjected to 30 minutes regional ischemia with no preceding ischemic preconditioning stimulus. Experiments commenced with 60 minutes perfusion for equilibration, after which regional ischemia was induced by occluding the left anterior descending coronary artery for 30 minutes, followed by

release of the occluding ligature and 120 minutes reperfusion (detailed description see Chapter Three).

Ischemic Preconditioning: Experiments represent those in which hearts from rats that were fed any one of the diets were subjected to the ischemic preconditioning protocol prior to 30 minutes regional ischemia. This consisted of 30 minutes equilibrium perfusion, three cycles of five minutes global ischemia, each followed by five minutes reperfusion before the onset of 30 minutes regional ischemia, then release of the occluding ligature before 120 minutes reperfusion (detailed description see Chapter Three).

5.2.1 Data handling and statistical analyses

Comparison was made between dietary groups and treatment groups with two-way analysis of variance (ANOVA) for diet and treatments (normoxic perfusion, IPC, control) main effect and diet x treatment interaction, and by multi-way ANOVA for diet, treatment and ischemic versus non-ischemic main effects with diet x ischaemia x treatment interaction. Tukey's test was used for post-hoc pairwise comparison of individual means and interactions. Results of significant interactions (such as illustrated in table 6.1A) are not specifically presented in graphs and tables but are presented as the results of further individual pairwise comparisons (as shown in table 6.1C). Linear regression was used to determine the relationship between measurements. All statistical analyses were performed using SPSS v.11.5 (SPSS Inc., Chicago, IL, USA) or Statistix (version seven) (Analytical software, Tallahassee, FL,USA) with significance accepted at $P<0.05$.

The percent of isolated hearts exhibiting ventricular tachycardia or ventricular fibrillation during ischemia or reperfusion were compared for dietary or ischemic preconditioning effect by Fisher's exact test.

5.3 RESULTS

5.3.1 Effects of diet on heart function during normoxic protocol

Under isovolumic conditions in normal heart, compared with the SF and (n-6) PUFA hearts, the (n-3) PUFA hearts exhibited significantly lower coronary flow ($P<0.001$) (Figure 5.1A), spontaneous heart rate (Figure 5.1B) that was ≥20 beats.min^{-1} lower ($P<0.01$) and ≥30 mmHg greater developed pressure ($P<0.01$) (Figure 5.1C).

These measures of heart function were not significantly different between the SF and (n-6) PUFA hearts. The end diastolic pressure, which was initially set at 6-8mmHg, gradually increased during equilibration in the SF and (n-6) PUFA hearts but remained significantly lower in the (n-3) PUFA hearts (Figure 5.1D). The maximum rate of ventricular relaxation was significantly greater in (n-3) PUFA (Figure 5.1E) ($p<0.01$) than the SF or (n-6) PUFA hearts. The maximum rate of ventricular pressure development (Figure 5.1F) and the rate-pressure product (Figure 5.1G), which is the product of heart rate and systolic pressure, were not significantly different between dietary groups.

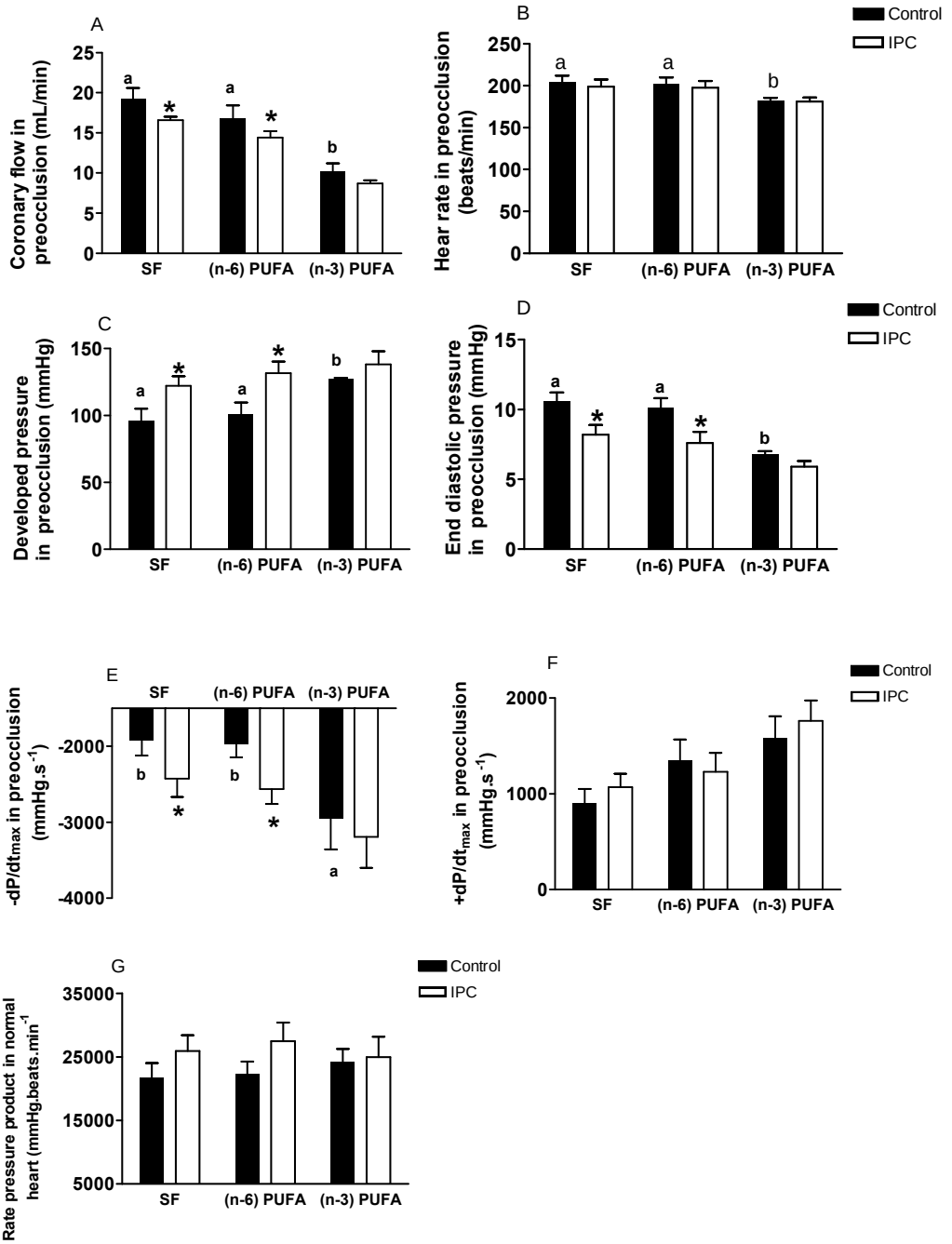


Figure 5.1 Measures of heart function at pre-regional ischemic time point prior to induction of the major ischemic episode. Coronary flow (A), heart rate (B), left ventricular developed pressure (C) end diastolic pressure (D), maximum rate of relaxation (E), maximum rate of contraction (F), and rate pressure product (G) in isolated hearts from rats fed SF, (n-6) PUFA and (n-3) PUFA diets for six weeks. Filled bar shows control hearts, open bar shows ischemic preconditioned (IPC) hearts. Values are means±SEM, n=6-9. * IPC and control groups differ, $p<0.05$. Within control groups, a variable without a common letter differs between diets, $p<0.05$.

5.3.2 Effects of ischemic preconditioning on heart function at pre-regional ischemic time point

There were no significant differences between ischemia-preconditioned and control hearts in the (n-3) PUFA group for any measurements of heart function. In SF and (n-6) PUFA hearts subjected to three cycles of 5-minute periods of global ischemic preconditioning, coronary flow was significantly lower compared to control SF and (n-6) PUFA hearts (Figure 5.1A) ($P<0.05$).

Spontaneous heart rate tended to be lower in preconditioned hearts but the differences did not reach significance (Figure 5.1B). Left ventricular developed pressure was significantly greater in the SF and (n-6) PUFA preconditioned hearts than in their respective controls (Figure 5.1C) ($P<0.05$). Left ventricular end diastolic pressure was significantly lower in preconditioned hearts compared to control SF and (n-6) PUFA hearts (Figure 5.1D) ($P<0.05$). The maximum rate of relaxation in preconditioned hearts was significantly higher compared to SF and (n-6) PUFA control hearts (Figure 5.1E) ($P<0.05$). There were no significant effects of ischemic preconditioning on maximum rate of contraction (Figure 5.1F) and rate pressure product (Figure 5.1G).

5.3.3 Effects of regional ischemia and reperfusion on heart function

Occlusion of the left anterior descending coronary artery produced regional ischemia and reduced coronary flow in all hearts (Figure 5.2A-C). Measures of heart function such as left ventricular developed pressure (Figure 5.2D-F), heart

rate (Figure 5.3), maximum rates of pressure development and relaxation (Figure 5.4) and rate pressure product (Figure 5.5) were significantly reduced in all hearts during ischemia

Effects of diet during reperfusion (control)

Occlusion of the left anterior descending coronary artery reduced total coronary flow in all dietary groups by a similar percentage, leaving residual flows of 78.2 ± 2.1% [(SF)]; 70 ± 3.1%, [(n-6) PUFA]; and 71 ± 2.2% [(n-3) PUFA] in each of the dietary control groups (Figure 5.2A-C).

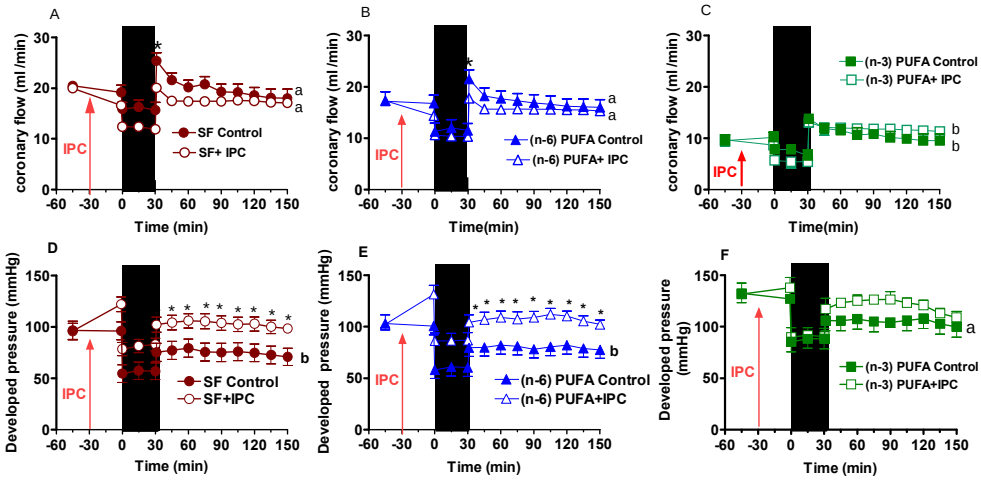


Figure 5.2 Effect of coronary occlusion (30-min regional ischemia) and reperfusion (120 minutes) on coronary flow (A-C) and left ventricular developed pressure (D-F) in control and ischemic preconditioned –isolated perfused hearts from rats that consumed the SF, (n-6) PUFA and (n-3) PUFA for six weeks. Filled bar on time-axes shows 30 minutes ischemia duration. Values are means±SEM, n=6-9. * IPC (ischemic preconditioning) and control groups differs at that time, $p<0.05$. The letter shown at the end of each curve represents all individual data points. Within control or IPC groups, curves for a variable without a common letter differs between diets, $p<0.05$.

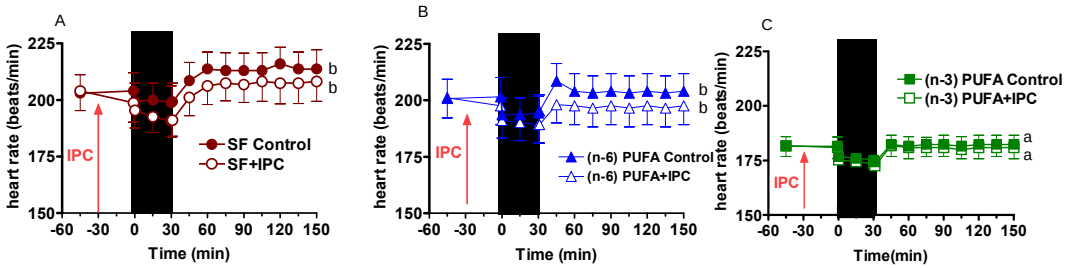


Figure 5.3 Effect of coronary occlusion (30-min regional ischemia) and reperfusion (120 minutes) on heart rate (A-C) in control and ischemic preconditioned –isolated perfused hearts from rats that consumed the SF, (n-6) PUFA and (n-3) PUFA for six weeks. Filled bar on time-axes shows 30-minutes ischemia duration. Values are means±SEM, n=6-9. The letter shown at the end of each curve represents all individual data points. Within control or IPC groups, curves for a variable without a common letter differs between diets, $p<0.05$.

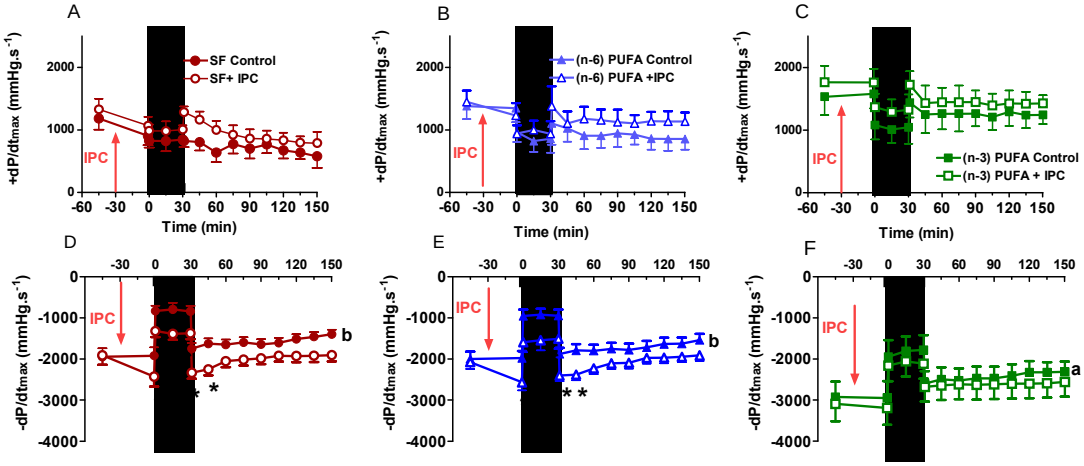


Figure 5.4 Effect of coronary occlusion (30-min regional ischemia) and reperfusion (120 minutes) on maximum rate of pressure development $+dP/dt_{max}$ and maximum rate of relaxation $-dP/dt_{max}$(A-F) in control and ischemic preconditioned –isolated perfused hearts from rats that consumed the SF, (n-6) PUFA and (n-3) PUFA for six weeks. Filled bar on time-axes shows 30 minutes ischemia duration. Values are means±SEM, n=6-9. * IPC (ischemic preconditioning) and control groups differs at that time, $p<0.05$. The letter shown at the end of each curve represents all individual data points. Within control or IPC groups, curves for a variable without a common letter differs between diets, $p<0.05$.

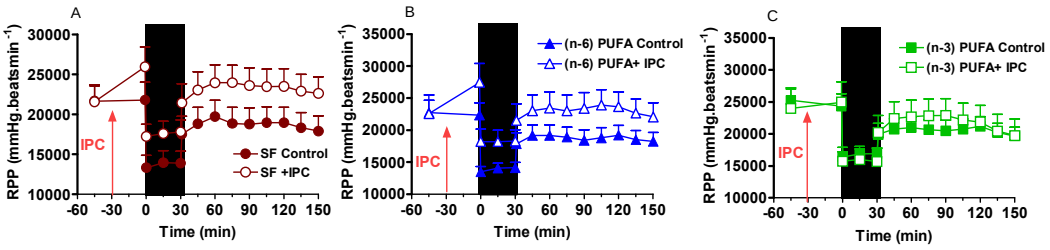


Figure 5.5 Effect of coronary occlusion (30-min regional ischemia) and reperfusion (120 minutes) on rate pressure product in control and ischemic preconditioned –isolated perfused hearts from rats that consumed the SF, (n-6) PUFA and (n-3) PUFA for six weeks. Filled bar on time -axes shows 30 minutes ischemia duration. Values are means±SEM, n=6-9.

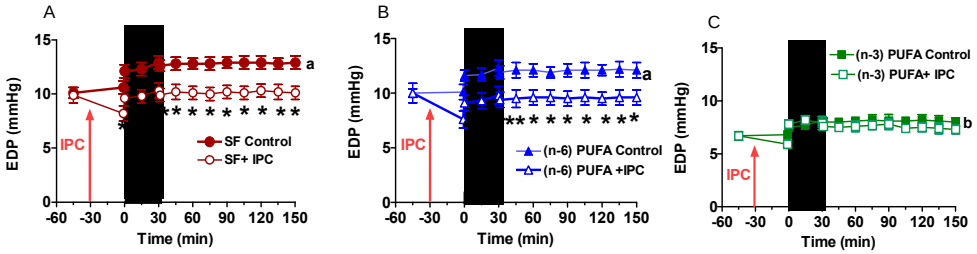


Figure 5.6 Effect of coronary occlusion (30-min regional ischemia) and reperfusion (120 minutes) on Left ventricular end diastolic pressure (A-C) in control and ischemic preconditioned –isolated perfused hearts from rats that consumed the SF, (n-6) PUFA and (n-3) PUFA for six weeks. Filled bar on time-axes shows 30-minutes ischemia duration. Values are means±SEM, n=6-9. * IPC (ischemic preconditioning) and control groups differs at that time, $p<0.05$. The letter shown at the end of each curve represents all individual data points. Within control or IPC groups, curves for a variable without a common letter differs between diets and a letter represents all each individual time points, $p<0.05$.

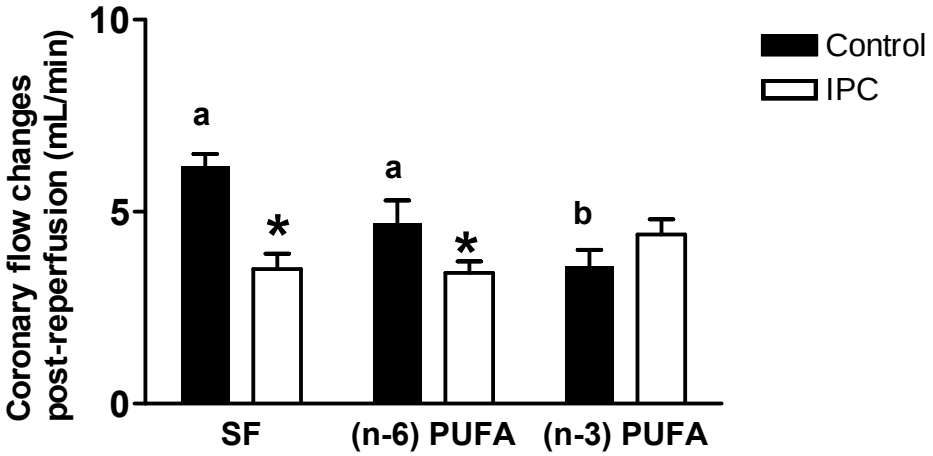


Figure 5.7 Increase in coronary flow one minute after termination of regional ischemia (pre-regional ischemic sample point) in control and IPC –isolated perfused hearts from rats that consumed the SF, (n-6) PUFA and (n-3) PUFA for six weeks. Values are means±SEM, n=6-9. * Shows difference between IPC (ischemic preconditioning) and control groups, $p<0.05$. Within control groups, a variable without a common letter differs between diets, $p<0.05$. Data derived from preocclusion and immediate post reperfusion values in Figure 5.2

During ischemia and reperfusion, the control (n-3) PUFA hearts exhibited significantly lower coronary flow (Figure 5.2C), significantly higher developed pressure (Figure 5.2F), and significantly lower spontaneous heart rate (Figure 5.3C) compared with the control SF and (n-6) PUFA hearts ($P<0.01$), which were not significantly different to each other. The maximum rate of ventricular relaxation was also greater in (n-3) PUFA than the SF and (n-6) PUFA groups (Figure 5.4) ($P<0.01$). The rate pressure product was not significantly different between dietary groups during ischemia and reperfusion (Figure 5.5). The end diastolic pressure remained significantly lower in the (n-3) PUFA hearts during ischemia and reperfusion (Figure 5.6C) than in SF (Figure 5.6A) or (n-6) PUFA (Figure 5.6B) hearts ($P<0.01$). The incidence of the arrhythmias, ventricular tachycardia and ventricular fibrillation, during both ischemia and reperfusion was significantly

lower in (n-3) PUFA hearts than in the SF hearts ($P<0.05$) (Table 1). The duration of arrhythmia episodes in (n-3) PUFA hearts also tended to be lower but the very low incidence prevented statistical comparison. The cumulative arrhythmia score was significantly lower in (n-3) PUFA control hearts compared with either SF and (n-6) PUFA control hearts in ischemia ($P<0.05$) and reperfusion ($P<0.05$) and (n-6) PUFA arrhythmia score was significantly lower than SF in ischemia ($P<0.05$) (Table 1)

Effects of diet and ischemic preconditioning during reperfusion

Ischemic-preconditioning significantly influenced cardiac function during reperfusion with lower coronary flow ($P<0.05$) (Figure 5.2A-C) and higher developed pressure (Figure 5.2D-F) compared to control hearts. These changes were observed in the SF and (n-6) PUFA hearts but not in the (n-3) PUFA hearts. For example, the developed pressure remained higher during ischemia in SF and (n-6) PUFA preconditioned hearts compared their control hearts. In the ischemic preconditioned hearts, developed pressure during reperfusion recovered to a level not significantly different to the equilibrium levels (-45min). There were no significant differences in developed pressure during ischemia or reperfusion between ischemic preconditioned and control (n-3) PUFA hearts ($P>0.05$). Ischemic preconditioning had no affect on heart rate (Figure 5.3). Ischemic preconditioning caused an increase in maximum rate of ventricular relaxation (Figure 5.4) and did not have any affect on rate pressure product in any of the dietary groups (Figure 5.5). Less rise in end diastolic pressure was seen in ischemic preconditioned SF and (n-6) PUFA hearts (Figure 5.6) ($P<0.05$). The incidence of arrhythmias, ventricular tachycardia and ventricular fibrillation, in

ischemia or reperfusion tended to be lower after preconditioning but did not reach significance (P=0.27-0.32). The episodes of ventricular tachycardia or ventricular fibrillation were of shorter duration (P<0.05) following ischemic preconditioning, and cumulative arrhythmia scores were significantly lower in both ischemia (P<0.05) and reperfusion (P<0.05) after ischemic preconditioning (Table 1).

Table 5.1 Effects of IPC on arrhythmia during ischemia and reperfusion in isolated hearts from rats fed SF, (n-6) PUFA, or (n-3) PUFA diets for six weeks

		Control hearts (no IPC)						IPC hearts					
	Diet	n	%VT	Duration of VT ,s	%VF	Duration of VF ,s	AS	n	%VT	Duration of VT ,s	%VF	Duration of VF ,s	AS
Ischemia	SF	9	89[a]	39.6 ±5.5	78 [a]	38.7 ±7.1	4.8 ±0.4 [a]	9	67 [a]	24.0 ± 2.1[*]	44	12.7 ±0.8	3.5 ±0.2[*a]
	(n-6) PUFA	9	67 [a,b]	18.5 ±3.4	44 [a,b]	12.3 ±3.4	3.2 ±0.3 [b]	9	44 [a,b]	7.8 ± 0.8[*]	33	1.3 ±0.3	[#]2.1 ±0.3[*b]
	(n-3) PUFA	9	22[b]	[ø] 9.0 ±7.0	0 [b]	[ø] n.d.	2.0 ±0.0 [c]	9	11 [b]	2.0 ± 0.0 [ø]	0	n.d. [ø]	1.0 ±0.0[c]
	All	27	59	27.8 ±4.3	41	28.0 ±6.0	3.8 ±0.3	27	41	16.0 ± 3.0[*]	30	7.0 ±2.2[*]	3.0 ±0.2[*]
Reperfusion	SF	9	89 [a]	20.1 ±4.0 [a]	67 [a]	29.8 ±6.0	4.8 ±0.5 [a]	9	56	14.0 ± 1.8[*]	44	9.8 ±2.4[*]	3.0 ±0.2[*a]
	(n-6) PUFA	9	78 [a,b]	18.8 ±0.7 [a,b]	33 [a,b]	16.0 ±4.5	3.7 ±0.4 [a]	9	56	6.4 ± 1.2[*]	22	7.5 ±2.5[*]	*2.0 ±0.2[*b]
	(n-3) PUFA	9	22 [b]	2.5 ±0.5 [b]	11 [b]	[ø] 5.0	2.0 ±0.0 [b]	9	11	4.0 ± 0.0 [ø]	11	4.0 ±0.0 [ø]	1.0 ±0.0[c]
	All	27	63	17.5 ±2.3	37	23.2 ±4.6	3.9 ±0.3	27	41	9.6 ± 1.5[*]	26	8.2 ±1.6[*]	2.3 ±0.2[*]

Values are means ± SEM, n=9 or 27 (all) or percentage. * Different from control in that diet group, $p<0.05$. Within ischemia or reperfusion, diet groups without a common letter differ, $p<0.05$. n.d. not detected. ø: incidence of VT or VF too low to conduct statistical analysis on duration. AS: arrhythmia score. "All" represents the collective incidence and duration of arrhythmias for all rats under control or IPC conditions.

5.4 DISCUSSION

The present study demonstrates that when rat hearts were subjected to prolonged occlusion of a major coronary artery, dietary (n-3) PUFA protected against ischemia induced dysfunction. Ischemic preconditioning also protected the rat heart against ischemia induced dysfunction but this was only observed in SF and (n-6) PUFA hearts. Ischemic preconditioning did not have any effect on further reducing ischemia induced dysfunction in (n-3) PUFA hearts.

In the early stages of regional myocardial ischemia, before cellular damage progresses to necrosis (infarction), heart function becomes depressed (especially diastolic relaxation and filling) (Opie, L.H. 2004). Characteristically, ventricular end diastolic pressure gradually rises, relaxation rate decreases as energy dependent processes decline and hearts become stiff and less amenable to relaxation. A high proportion of hearts become arrhythmic. When blood flow is restored, heart function recovers with partial restoration of these parameters towards normal. In the present study, the characteristic ischemia-induced rise in end diastolic pressure and arrhythmia were less pronounced in (n-3) PUFA hearts and functional recovery during subsequent reperfusion was enhanced relative to SF and (n-6) PUFA hearts. Similarly in ischemic preconditioned hearts, the end diastolic pressure remained low during ischemia and reperfusion, arrhythmias were less pronounced and functional recovery was enhanced, but this was only evident in the SF and (n-6) PUFA hearts. This confirms earlier reports that both ischemic preconditioning and (n-3) PUFA reduce the severity of reperfusion arrhythmias and enhance the recovery of contractile function of the

myocardial region-at-risk in isolated heart models (Pepe & McLennan, P.L. 1996; Shiki, K. & Hearse, D.J. 1987). However, while both (n-3) PUFA and ischemic preconditioning protected the heart against the adverse effects of prolonged ischemia, the effects were not additive or synergistic. Indeed, in terms of arrhythmia generation, diastolic relaxation and end diastolic pressure, fish oil feeding apparently produced greater cardioprotection than acute ischemic preconditioning alone.

Myocardial relaxation during cardiac diastole is critical to the adequate filling of the ventricle and hence cardiac output. Relaxation is mediated by several cellular processes, including Ca^{2+} reuptake into the sarcoplasmic reticulum and Ca^{2+} extrusion from the cell via Na^{+}-Ca^{2+} exchange, therefore relaxation is often retarded by impaired calcium removal from the vicinity of the contractile proteins (Gao, W.D. *et al.* 1995). The better maintenance of maximum rate of relaxation and prevention of the rise in end diastolic pressure by either ischemic preconditioning or dietary fish oil in the present study suggests altered calcium handling during ischemia, thus reducing the rise in the concentration of cytosolic calcium.

The low resting heart rate observed with (n-3) PUFA in present study was previously reported in other isolated heart studies (Demaison, L. *et al.* 2000; Pepe & McLennan, P.L. 1996) and in humans (Buckley, J.D. *et al.* 2008; Dallongeville, J. *et al.* 2003; Geelen, A. *et al.* 2005; Mozaffarian, D. *et al.* 2005b). The calcium cycling and the rhythmic spontaneous calcium release from sarcoplasmic reticulum play an important role in the initiation and regulation of normal heart rate (Lakatta, E.G. *et al.* 2008; Yaniv, Y. *et al.* 2008) and reduced intracellular calcium concentrations are associated

with reduced or slower spontaneous sarcoplasmic reticulum calcium release and lower heart rate. Although (n-3) PUFA are postulated to affect the sympathetic and parasympathetic nervous systems and influence heart rate through their interaction with the adrenergic system (Dallongeville, J. *et al.* 2003), the *in vitro* nature of the present study isolates these effects to the influence of membrane incorporation. The experimental conditions therefore exclude changes in autonomic nervous function or peripheral vascular effects that could contribute *in vivo* to reduce heart rate (Grimsgaard, S. *et al.* 1998). Low heart rate is associated with reduced cardiovascular risk (Ferrari, R. *et al.* 2008; Fox, K. *et al.* 2008) including reduced risk of sudden death (Jouven, X. *et al.* 2001).

The reduced risk of sudden cardiac death associated with fish or fish oil intake is usually attributable to fatal cardiac arrhythmia in ischemia or reperfusion. Such arrhythmia were reduced by both IPC and fish oil in this study. The precise mechanism of antiarrhythmic effects of ischemic preconditioning (Hagar, J.M. *et al.* 1991; Shiki, K. & Hearse, D.J. 1987) or dietary fish oil (Hirafuji, M. *et al.* 2003; Nair, S.S. *et al.* 1997) is still not clear. However, it has been suggested that both ischemic preconditioning (Argaud, L. *et al.* 2004) and (n-3) PUFA (Hirafuji, M. *et al.* 2003; Nair, S.S. *et al.* 1997; Pepe, S. *et al.* 1999) may attenuate ischemia-induced electrophysiological effects such as spontaneous depolarization associated with intracellular rise in calcium. A close relationship exists between elevated intracellular concentration of calcium and generation of arrhythmia (Brooks, W.W. *et al.* 1995; Kihara, Y. & Morgan, J.P. 1991) as seen with ventricular relaxation and heart rate. A slow spontaneous release of calcium from

sarcoplasmic reticulum during diastole enhances the concentration of free cytoplasmic calcium and stimulates Na^{+}-Ca^{2+} exchange current, the intracellular calcium -induced current and calcium induced CI^{-} current. All these changes slow the rate of depolarization that underpins spontaneous heart rate. When depolarization reaches threshold for a generative increase in Na^{+} conductance, an extra systole is generated that may become self-replicating, resulting in runs of successive action potentials (Carmeliet, E. 1999) and manifested as repetitive arrhythmia such as VT or VF. It has been reported that Ca^{2+} sparks associated with spontaneous release of Ca^{2+} from the sarcoplasmic reticulum have the potential to locally raise the sarcolemmal membrane potential to threshold for action potential generation and arrhythmia. These calcium sparks are reduced following either acute application of (n-3) PUFA (Honen, B.N. *et al.* 2003; O'Neill, S.C. *et al.* 2002) or after fish oil pre-feeding (Honen, B.N. *et al.* 2003). Similarly, the rise in intracellular Ca^{2+} concentrations and cellular Ca^{2+} transients initiated by various stimuli are inhibited in neonatal cardiomyocytes after 3-5 day (n-3) PUFA incubation and in adult cardiomyocytes after fish oil pre-feeding (Hallaq, H. *et al.* 1990; Leifert, W.R. *et al.* 2001), implying that (n-3) PUFA incorporation into membranes has the ability to prevent the rise in intracellular calcium concentration.

Why is control of cellular calcium handling important to normal function of the myocytes? In resting conditions, the concentration of calcium in the cytosol is much lower than the concentrations found in the extracellular medium. Within the cell, calcium is stored largely in sarcoplasmic reticulum, keeping the cytosolic

concentration low. Intracellular calcium concentration is tightly regulated by sarcoplasmic reticulum and sarcolemmal membrane calcium ATPases and the Na^{+}- Ca^{2+} exchanger (Figure 5.7). The concentration gradient from outside to inside the cell allows influx of calcium into myocardial cells upon opening of plasma membrane channels. The normal cardiac cycle involves the sequential activation of membrane calcium channels by the cardiac action potential, entry of calcium down its concentration gradient to stimulate calcium-induced calcium release from the sarcoplasmic reticulum stores. This increase in cytosolic calcium produces conformational changes in contractile proteins allowing them to bind and contract cell. Contraction is sustained until calcium is removed from the cytosol. Mitochondria accumulate part of the calcium that is not rapidly restored to sarcoplasmic reticulum stores between beats, therefore, acting as a fixed buffer (Pinton, P. *et al.* 1998). Slight increases in mitochondrial calcium stimulate Krebs cycle enzymes and ATP synthesis in the myocardium (Ferrari, R. *et al.* 1993), when intracellular calcium increases above a certain threshold, mitochondrial calcium overload occurs (Mettauer, B. *et al.* 2006) because the removal mechanisms become saturated whereas, the uniporter, responsible for calcium uptake does not saturate, and calcium continues to accumulate leading to mitochondrial dysfunction (Giorgi, C. *et al.* 2008). Excess calcium accumulated during ischemia and reperfusion also excessively activate protein kinases and cause damage to myocardial cells. Excess intracellular calcium interfering with mitochondrial function is also implicated in energetic decline in heart failure (Mettauer, B. *et al.* 2006).

During ischemia and reperfusion, calcium regulatory processes break down, leading to direct entry of calcium into myocardial cells via voltage -dependent channels (Steenbergen, C. *et al.* 1990). intracellular acidosis and Na^+ accumulation increases subsequent Ca^{2+} entry via Na^+- Ca^{2+} exchanger (Grinwald, P.M. 1982; Tani, M. & Neely, J.R. 1989) and free cytosolic calcium is partly redistributed to intracellular stores such as the mitochondria and sarcoplasmic reticulum (Krause, S.M. *et al.* 1989). The resultant excessive build up of calcium in the cytoplasm and especially mitochondria is known as calcium overload.

The (n-3) PUFA may directly interact with calcium regulatory enzymes in cardiomyocytes promoting removal from the cell, reducing the concentration of intracellular calcium and preventing cellular calcium overload (Siddiqui, R.A. *et al.* 2008). Isolated cell models provide consistent evidence that ion channel –mediated effects of (n-3) PUFA can have antiarrhythmic effects (Leaf, A. 2001; Leaf, A. *et al.* 2003). Acute administration of (n-3) PUFA to isolated myocytes via added fatty acids inhibit voltage-gated sodium channels (Leaf, A. 2001; Leaf, A. *et al.* 2003; Leifert, W.R. *et al.* 2001) and L-type calcium channels, however only the latter has been confirmed through dietary studies (Leifert, W.R. *et al.* 2001). As this is the major pathway for calcium influx into cardiomyocytes, it implies a mechanism for (n-3) PUFA preventing calcium overload during stress and eliciting an increased electrical stability of the myocardium (Leaf, A. *et al.* 1999; Leaf, A. 2001). The (n-3) PUFA have been shown to increase by approximately 50%, the electrical threshold required to induce an action potential as well as prolonging the refractory period after an action potential by three-fold and may thereby reduce electrical excitability is isolated cells

(Kang *et al.* 1995b) and the vulnerability to ventricular fibrillation (Kang *et al.* 2000). Dietary fish oil also raises electrical excitability and VF threshold (McLennan, P.L. *et al.* 1992a, 1993). Low-voltage-activated T-type (transient type) Ca^{2+} channels regulate spontaneous electrical activity and Ca^{2+} entry in excitable cells, such as neurons and cardiac myocytes and play a pacemaker role (Perez-Reyes, E. 2003). It was also reported that short term application of (n-3) PUFA can reduce T-type calcium entry in adrenal glomerulosa cells, and thereby also elicit electrical stability in that tissue (Danthi, S.J. *et al.* 2005). Many of these effects have only been recorded following acute administration of fatty acids. Until they can be demonstrated after compositional change in the membranes, there must remain some doubt about their individual contribution to dietary effects.

The sarcoplasmic reticulum is the source of most cellular calcium that activates myofilaments (Fabiato, A. & Fabiato, F. 1975). Sarcoplasmic calcium release can be disordered to cause arrhythmias due to overload or to defects in the ryanodine receptor channel which is responsible for calcium release. Studies suggested that decrease in activation of ryanodine receptor calcium release channel-II and calcium –calmodulin dependent kinase II induced by feeding (n-3) PUFA may be one mechanism responsible for alterations in ionic conductance and arrhythmia generation after (n-3) PUFA intake (Zaloga, G.P. *et al.* 2006).

Delayed afterdepolarizations are the changes in membrane potential following a normal action potential and are generated by spontaneous but abnormal release of calcium from the sarcoplasmic reticulum. Increased sodium-calcium exchanger

activity leading to increased intracellular calcium and spontaneous release of calcium from the sarcoplasmic reticulum. These are believed to contribute to the occurrence of delayed afterdepolarization, and can trigger arrhythmic events (Verkerk, A.O. *et al.* 2006).

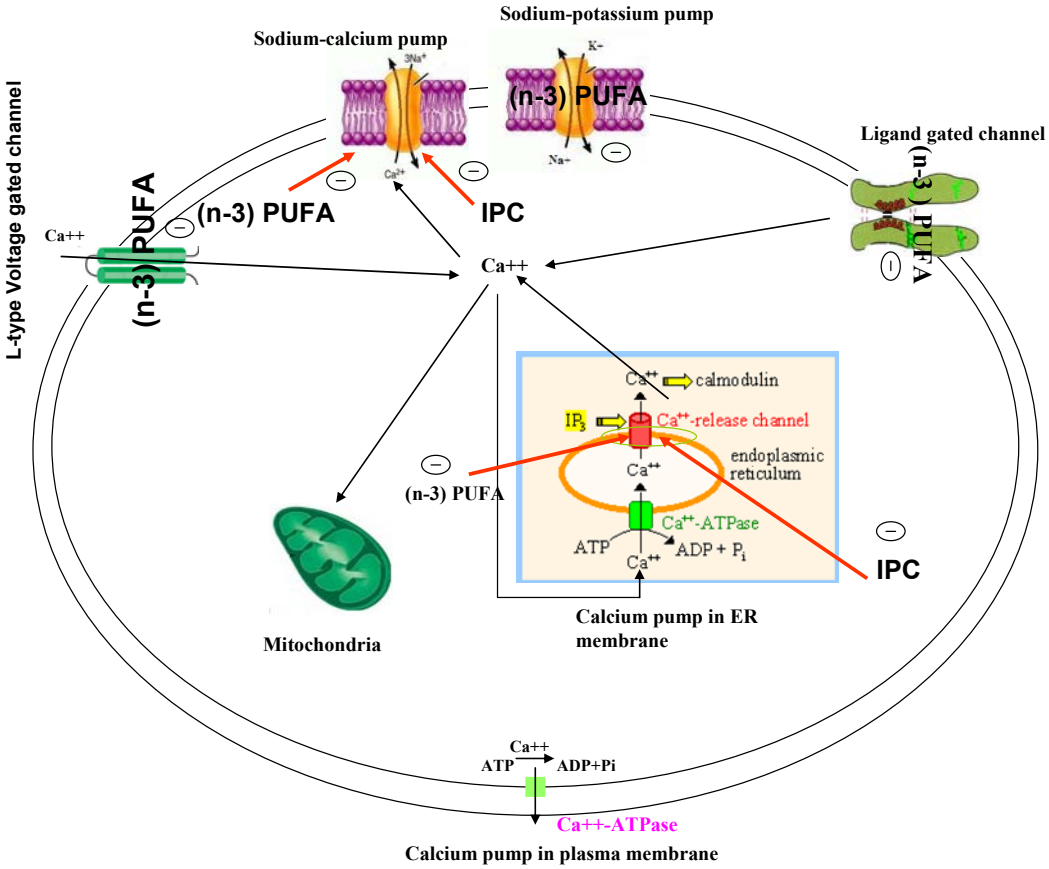


Figure 5.7 Effects of (n-3) PUFA and IPC on intracellular calcium regulation. The calcium enters cardiomyocytes mainly through L-type calcium channels and ligand-gated channels after hormonal or mechanical stimulation. The calcium in sarcoplasmic reticulum is then released in response to calcium stimulation through ryanodine-sensitive calcium channels for utilization of cellular regulation process. The unutilized calcium is then sequestered from the cytosol by calcium ATPase pump and stored in sarcoplasmic reticulum. The concentration of Na^+ is then balanced by Na^+-K^+ pump. The (n-3) PUFA and IPC inhibit rises in intracellular calcium concentrations by acting on several cites (shown as negative marks). Modified from (Siddiqui, R.A. *et al.* 2008)

A dietary supplementation with (n-3) PUFA in pigs resulted in 60% reduction in sodium-calcium exchanger current (Verkerk, A.O. *et al.* 2006). Therefore, sodium-calcium exchanger current is another possible mechanism that could be responsible for alterations in ionic conductance and arrhythmia generation after (n-3) PUFA intake.

The (n-3) PUFA have also been shown to influence major classes of phospholipases that also play important roles in calcium mobilization. Activation of phospholipase C (PLC) contributes to several processes believed to be involved in arrhythmia including the formation of the putative calcium-releasing compound inositol 1,4,5-trisphosphate (IP_3) and the activator of the PKC isoenzymes,1,2-diacylglycerol (DAG) (Berridge, M.J. 1997; De Jonge, H.W. *et al.* 1995; Nishizuka, Y. 1992). IP_3 binds to the ryanodine (RyR) receptor to induce an increase in intracellular calcium (Figure 5.8). The release of IP_3 following ischemia may therefore contribute to the generation of arrhythmias through promotion of calcium overload (Du, X.-J. *et al.* 1995; Woodcock, E.A. *et al.* 1996). Interestingly, ischemic preconditioning was shown to block this postischemic burst of IP_3 in isolated heart (Anderson, K.E. & Woodcock, E.A. 1995) (Figure 5.8), and both IP_3 and arrhythmia production in ischemia are blocked by dietary (n-3) PUFA (Anderson, K.E. *et al.* 1996). Reduced IP_3 release also occurs in cardiac myocytes from fish oil-fed pigs (Nair, S.S. *et al.* 2000) and EPA treated cells (de Jonge, H.W. *et al.* 1996). Pharmacological inhibition of PLC improves myocardial function recovery after ischemia-reperfusion (Asemu, G. *et al.* 2004) and reduces arrhythmia (Bian, J.S. *et al.* 1998) in isolated rat hearts. DHA was found to suppress PLC

activated by TNFα (Weber, C. *et al.* 1995) suggesting that the incorporation of (n-3) PUFA in the cell membrane phospholipids might be the mechanism by which (n-3) PUFA exert their effects on PLC (Salem, N. *et al.* 1988).

The (n-3) PUFA also are known to modulate another important phospholipid metabolising enzyme, phospholipase A_2. PLA_2 is involved in the activation of calcium channels via generation of lysophospholipids (Smani, T. *et al.* 2004). Most of (n-3) PUFA effects on PLA_2-mediated signalling events may be related to the fatty acyl composition of phospholipids, the PLA_2 substrate (Grynberg, A. *et al.* 1992). The (n-3) PUFA are incorporated into membrane phospholipids on sn-2 position replacing arachidonic acid and can block PLA_2 effects by inhibiting production of proinflammatory arachidonic acid derived eicosanoids. Therefore, the (n-3) PUFA containing phospholipids alter phospholipase activities and also alter the generation of phospholipase-derived signalling molecules. This is one of the mechanisms whereby (n-3) PUFA may elicit their antiarrhythmic effects and their prevention of impaired relaxation and elevated end diastolic pressure, principally through their ability to reduce calcium overload through affects on PLC or PLA_2, and their downstream products IP_3 and DAG or lysopholipids and free fatty acids. Some of these properties appear to be shared by ischemic preconditioning.

The (n-3) PUFA may also play a role in modulating kinase–mediated serine/threonine and tyrosine phosphorylation of cellular proteins. For example, calcium channel activity is modulated by serine/threonine phosphorylation by cAMP-dependent protein kinase and PKC (Kuriyama, H. *et al.* 1995; Levitan, I.B. 1994). A non-

receptor c-src tyrosine kinase, was found to be associated with calcium channels in smooth muscle cells (Hu, X.-Q. *et al.* 1998). The (n-3) PUFA have been found to inhibit src-mediated signaling (Chen, W. *et al.* 2007) (Figure 5.8). The activation of ras, raf-1 and the MAP kinase cascade is the downstream signaling of c-src (Parsons, J.T. & Parsons, S.J. 1997). MAP kinase is known to phosphorylate load-induced p^{90rsk} kinase in cardiac myocytes (Yamazaki, T. *et al.* 1993). It was reported that DHA in rat cardiomyocytes inhibits ras-mediated raf-1-MAP kinase- p^{90rsk} activation pathway, thereby eliciting its anti-arrhythmic effect (Siddiqui, R.A. *et al.* 2004) (Figure 5.8).

Studies indicate that (n-3) PUFA also affect a small G-protein, Rho which mediates the increased myosin light chain (MLC) activity (Sah, V.P. *et al.* 1996). Rho kinase was identified as one of the putative downstream effectors of Rho (Matsui, T. *et al.* 1996). MLC and the myosin binding subunit of MLC phosphatase are the substrates of Rho kinase (Amano, M. *et al.* 1996). The sensitivity of myosin to calcium in cardiomyocytes can be altered by the phosphorylation of MLC and MLC phosphatase (Kureishi, Y. *et al.* 1997). Speculations exist that (n-3) PUFA may alter translocation of Rho and its interaction with Rho kinase, thereby elicit electrical stability (Adamson, P. *et al.* 1992) (Figure 5.8).

Protein kinase C is also affected by (n-3) PUFA. Conventional PKC isoenzymes are calcium dependent, having three domains, named DAG binding domain, calcium binding domain and catalytic domain (Meller, N. *et al.* 1998). Thus (n-3) PUFA may influence PKC by modulation of DAG or calcium in the cell as described above. Activation of PKC is associated with genesis of arrhythmia, intracellular calcium homeostasis and properties of contractile proteins, ion conductance and transport

activity. For example, PKC activation is observed concurrently with increases in calcium and generation of DAG as a result of PLC activity (Mellor, H. & Parker, P.J. 1998) and load-induced p^{70rsk} kinase is activated via a PKC pathway (Laser, M. *et al.* 1998).

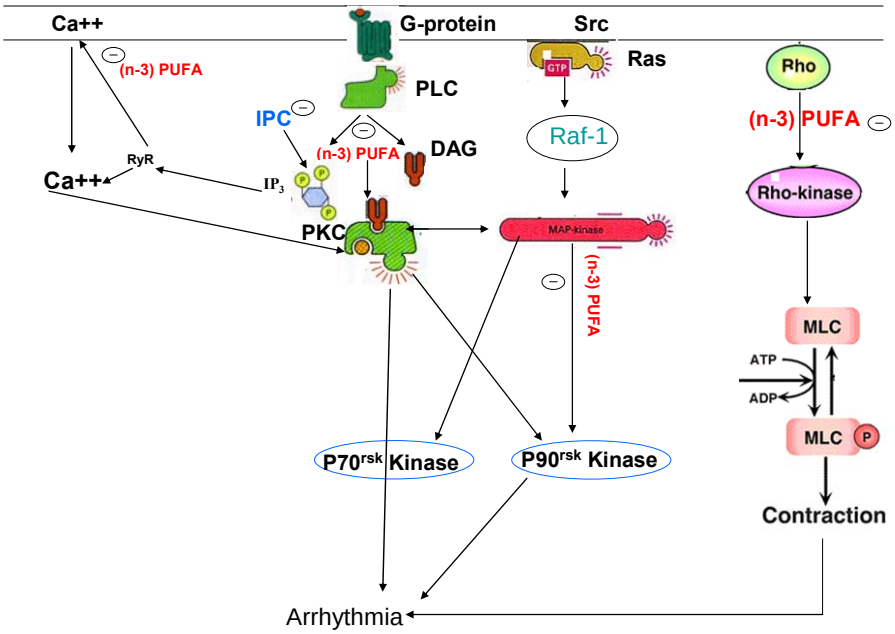


Figure 5.8 Protein kinase targets of (n-3) PUFA and IPC for regulating cardiovascular functions. The (n-3) PUFA prevent an increase in cytosolic calcium by directly affecting G- protein mediated activation of phospholipase C and subsequent generation of inositol IP_3 and DAG. The inositol IP_3 also an important pathway of IPC on prevention a rise in cytosolic calcium. The (n-3) PUFA subsequently prevent the activation of PKC and its downstream load induced P^{70rsk} kinase. The (n-3) PUFA also affect tyrosine kinase and Raf-mitogen activated kinase pathways. As the result, affect protein synthesis through load induced P^{90rsk} In addition (n-3) PUFA prevent myofibrillogenesis by affecting Rho protein, Rho kinase and MLC phosphorylation. By affecting these pathways individually or collectively (shown as negative marks), (n-3) PUFA afford antiarrhythmic effect. Modified from (Siddiqui, R.A. *et al.* 2008)]

Evidence exists that (n-3) PUFA significantly alter the fatty acid composition of DAG in myocardium and inhibit PKC activity (Takahashi, R. *et al.* 2005). Incorporation of EPA into membrane phospholipids of vascular smooth muscle cells has also been

shown to inhibit PKC activity and intracellular calcium mobilization (Bordoni, A. *et al.* 1992; Nyby, M.D. *et al.* 2003). Similarly, DHA inhibited the activation of membrane-bound PKC in cardiac cells (Castillo, A. *et al.* 2005) (Figure 5.8). Since it is confirmed in this thesis and many other studies that DHA is accumulated in the hearts of fish oil fed rats (Pepe, S. & McLennan, P.L. 2002), the antiarrhythmic effect of (n-3) PUFA may be mediated by changes in PKC and PLC activity..

In addition to effects on the protein kinase PKC described above, activation of protein kinase A and calcium–calmodulin dependent kinase II is decreased by feeding (n-3) PUFA(Zaloga, G.P. *et al.* 2006). PKA is the major mediator of beta-adrenergic signaling and plays an important role in ion channel modulation linked to sudden death in humans (Pogwizd, S.M. & Bers, D.M. 2004). Calmodulin kinase II also has been shown to play an important role in arrhythmia susceptibility in mice and rabbits (Anderson, M.E. *et al.* 1998; Zhang, R. *et al.* 2005). Suppression of calmodulin kinase II activity can attenuate early afterdepolarization and this may be another mechanism responsible for alterations in ionic conductance and arrhythmia generation after (n-3) PUFA intake.

Considerable evidence, obtained mainly from rat isolated heart studies, has indicated that ischemic preconditioning also produces adaptations in calcium regulation. This is characterized by lower intracellular concentration of calcium after preconditioning episodes with repeated brief ischemia (Smith, G.B. *et al.* 1996), and reduced intracellular calcium concentrations during sustained, longer periods of ischemia (Murphy, E. *et al.* 1995; Steenbergen, C. *et al.* 1993; Tosaki, A.

et al. 1994; Wang, Y.G. *et al.* 2001). There fore, the prevention of cellular calcium overload is an important mechanism of cardioprotection in ischemic preconditioning.

The ryanodine channel on the sarcoplasmic reticulum is a source of internal calcium that is likely modified by ischemic preconditioning (Figure 5.7). Ischemic preconditioning in rat isolated hearts decreases the density of ryanodine binding sites and slows in the rate of calcium-induced calcium release(Zucchi, R. *et al.* 1995).. This would facilitate a delay in the accumulation of calcium during imposition of subsequent sustained ischemic episodes. Calcium uptake by the sarcoplasmic reticulum, normally elevated in the presence of ryanodine after sustained ischemia and reperfusion (Mubagwa, K. *et al.* 1997), was not increased in preconditioned hearts (Tani, M. *et al.* 1996). In addition, sarcoplasmic reticulum calcium release (Mubagwa, K. *et al.* 1997), was significantly reduced in both ischemia and reperfusion in the preconditioned heart (Tani, M. *et al.* 1996). This suggests that preconditioning inhibits an inappropriate release of calcium from the sarcoplasmic reticulum during sustained ischemia-reperfusion in the rat heart. In another example of potential commonality, there is also indirect evidence of (n-3) PUFA modulating ryanodine receptor activity responsible for sarcoplasmic reticulum calcium release to prevent arrhythmias in the isolated heart (Pepe, S. & McLennan, P.L. 2002)

Calcium influx may also be inhibited by ischemic preconditioning secondary to intracellular acidosis. The resultant in Na^+-H^+ exchange and influx of Na^+, and the

subsequent increase in Na-Ca^{2+} exchange (Figure 5.7) prevents cell death. Indeed, lower concentration of calcium are observed in preconditioned hearts accompanied by an attenuated acidosis and reduced level of Na^+ gain (Asimakis, G.K. *et al.* 1992; Murphy, E. *et al.* 1995; Steenbergen, C. *et al.* 1993; Tosaki, A. *et al.* 1994). Another potential site of action ischemic preconditioning cardioprotection involves the mitochondrial ATP-sensitive potassium channels (K_{ATP}) which have important cellular functions, including homeostasis maintenance and vascular tone regulation under physiological conditions. Ischemic preconditioning opens the mitochondrial K_{ATP} channel and releases calcium from the mitochondria into cytoplasm, thus decreasing basal mitochondria calcium concentrations (Holmuhamedov, E.L. *et al.* 1998) with the excess removed from the cytoplasm by normal processes. This increases the calcium buffering capacity allows mitochondria to take up more calcium, and at a slower rate, which reduces the deleterious effect of calcium overload (Holmuhamedov, E.L. *et al.* 1999). Again, a similar property has been described for (n-3) PUFA (Pepe, S. *et al.* 1999) which reduce inappropriate stimulation of calcium dependent pyruvate dehydrogenase and oxygen wasting by mitochondria. Oxygen consumption is reduced by blockage of mitochondrial calcium uptake or by membrane incorporation of (n-3) PUFA (Pepe, S. & McLennan, P.L. 2002).

From the above evidence, the cardioprotective effects of both ischemic-preconditioning and nutritional preconditioning with fish oil might be related to their ability to affect calcium influx, efflux or intracellular calcium redistribution, factors known to impair cardiac relaxation and promote cellular damage. A variety

of direct and indirect mechanisms of intracellular calcium modulation can be demonstrated with (n-3) PUFA.

In addition to intracellular sites of action for (n-3) PUFA and ischemic preconditioning, there is potential for variations in coronary blood supply to influence the responses to ischemia and reperfusion. While some reports suggest that the cardioprotective effects (n-3) PUFA may be due to altered blood platelet function or other circulatory factors (Zhu, B.Q. *et al.* 1994), neither blood platelets nor fatty acids were circulating in the isolated heart perfusate in the present study. Therefore, the cardioprotection afforded by (n-3) PUFA in this study must be localised within the myocardium following fatty acid incorporation into cellular membranes. In the isolated heart, where coronary perfusion pressure is held constant, coronary flow is entirely dependent on intrinsic vascular resistance. The effects of neither nutritional- nor ischemic-preconditioning were attributable to differences in degree of ischemia, or variations in collateral flow, since all hearts exhibited equivalent reductions in coronary flow during the regional ischemia. This implied a similar ischemic region in all hearts, which was subsequently confirmed in stained slices.

Myocardial ischemia, as produced by occlusion of the left anterior descending coronary artery, rapidly leads to depletion of glycogen stores and diminishing contractions (West, J. 1990). When blood flow is restored, vasodilation within the ischemic region leads to greater than normal blood flow, known as reactive hyperaemia. This compensatory increase in blood flow provides repayment of so called “flow-debt or oxygen debt” incurred during occlusion, potentially hastening

metabolic and functional recovery of post ischemic tissue (Loscalzo, J. & Vita, J.A. 1994). Reactive hyperaemia primarily depends on local production and accumulation of adenosine and other non–endothelium-dependent vasodilator metabolites during the period of obstructed blood flow, that dilate tissue microvessels (Loscalzo, J. & Vita, J.A. 1994; West, J. 1990). Although all groups of hearts displayed reactive hyperaemia at the start of reperfusion, this increase in post-ischemic blood flow was less evident in both (n-3) PUFA and ischemic preconditioned hearts, which indicates that they may adaptively reduce the oxygen demand of the heart and produce less adenosine accumulation from terminal breakdown of ATP. Coronary flow can be shown to have a nearly linear relationship with myocardial oxygen consumption within any heart (Braunwald, E. *et al.* 1957). The low coronary flow observed in (n-3) PUFA hearts, occurring without detriment to cardiac function, concurs with the low oxygen demand and high coronary flow reserve of (n-3) PUFA hearts (Pepe, S. & McLennan, P.L. 2002, 2007).

Underlying changes in mitochondrial metabolism may contribute to more efficient oxygen utilization in (n-3) PUFA hearts. In the face of increased energy demand, the activation of cardiac mitochondrial dehydrogenases, including pyruvate dehydrogenase, is believed to lead the maintenance of $NADH/NAD^+$ and the proton-motive force required for oxidative phosphorylation (Brandes, R. & Bers, D.M. 1997; Hansford, R.G. 1985). Calcium ions play a role to maintain a balance of energy supply and demand by activating dehydrogenases and generating ATP through oxidative phosphorylation (Hansford, R.G. 1985, 1991, 1994; Heineman, F.W. & Balaban, R.S. 1990). Calcium activated enzymes reside in the mitochondrial matrix,

and their activation requires the transmission of changes in cytosolic calcium into the mitochondria pathways (Gunter, T.E. & Pfeiffer, D.R. 1990). Dietary fat modulation by (n-3) PUFA can significantly prevent the inappropriate increase in concentration of mitochondrial calcium and catalytically active pyruvate dehydrogenase, and thus may contribute to more efficient oxygen utilization (Pepe, S. *et al.* 1999). The potential modulation of mitochondrial calcium levels by (n-3) PUFA was discussed above. Reduced rates of mitochondrial respiration and increased energy utilisation efficiency is similarly evident after ischemic-preconditioning (da Silva, M.M. *et al.* 2003; Monnet, X. *et al.* 2006; Tanoue, Y. *et al.* 2002). Ischemic preconditioning promotes partial inhibition of NADH-supported respiration and an increase in H_2O_2 release. This mild state of oxidative stress is an important signal in ischemic preconditioning because it prevents a larger increase in reactive oxygen species release after ischemia reperfusion by activating mitoK_{ATP} channels. The mitoK_{ATP} channels activity then leads to more efficient oxidative phosphorylation, lower mitochondrial ATP consumption, the prevention of mitochondrial calcium accumulation (Dos Santos, P. *et al.* 2002) and attenuated reperfusion reactive oxygen species release (Vanden Hoek, T. *et al.* 2000). Therefore, ischemic preconditioning most likely provides cardioprotection by improving myocardial oxygen handling and efficiency rather than having an effect on oxygen delivery. The role of oxidation and reactive oxygen species in the effects of ischemic preconditioning and (n-3) PUFA is considered in the next chapter.

In conclusion, the present study demonstrated that dietary fish oil induces a form of nutritional preconditioning, limiting ischemic cardiac injury, dysfunction and

endowing cardioprotection at least as powerful as ischemic preconditioning. Notably, ischemic preconditioning provided no additional protection in (n-3) PUFA hearts. In particular the principal consequences of ischemia attributed to intracellular calcium overload, namely cardiac arrhythmia and elevation in end diastolic pressure were modulated by (n-3) PUFA and ischemic preconditioning, as was resting heart rate, another factor modulated by intracellular calcium. The cardioprotection afforded by both ischemic preconditioning and (n-3) PUFA may share common pathways involved in calcium handling.

CHAPTER 6

EFFECT OF ISCHEMIC PRECONDITIONING AND FISH OIL ON OXIDATIVE STATUS IN ISOLATED RAT HEARTS.

6.1 INTRODUCTION

Oxidation-reduction reactions (redox reactions) are important in normal cell functioning. They are involved in processes such as fuel metabolism (Botham, K. *et al.* 2009C), cell signaling (Gabbita, S.P. *et al.* 2000), and inflammation (Sacheck, J.M. & Blumberg, J.B. 2001). Oxidation-reduction reactions are sources of free radicals. A free radical is defined as any atomic or molecular species capable of independent existence that contains one or more unpaired electrons in one of its molecular orbitals (Halliwell, B. *et al.* 1992). Free radicals that originate from oxygen are called reactive oxygen species. Reactive oxygen species, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-) are generated by an inefficient series of reactions that reduce oxygen to water through the electron transport chain (Radak, Z. 2000). Superoxide is also generated by intracellular enzymes as part of the inflammatory responses (Tiidus, P.M. 1998). Heart cells have a high intracellular oxygen content (Tamura, M. *et al.* 1978) and because of increased electron transport chain activity (Satoh, Y. & Shikama, K. 1981), these cells are constantly being exposed to oxidizing species. However, cells posses defence mechanisms, namely antioxidants,

to cope with these intermediates so that a redox balance is maintained. Either excessive oxidation or insufficient antioxidant defence can disrupt cellular redox balance and lead to oxidative stress, tissue damage and altered physiological function (Gabbita, S.P. *et al.* 2000).

It has been shown that oxygen attacks the double bond in fatty acids to form peroxide linkages (Frankel, E.N. 2005). Polyunsaturated fatty acids naturally contain double bonds. The bis-allelic hydrogen atoms on the methylene carbon atoms between two double bonds are particularly vulnerable. Therefore, membrane phospholipids which contain a high content of unsaturated fatty acids are more susceptible to oxidation (Frankel, E.N. 2005). Membrane lipid peroxidation is a chain reaction event in which additional reactive species, generated by the reactive oxygen species attack, cause further damage to neighbouring lipids and other molecules (Beckman, K.B. & Ames, B.N. 1998). The process is initiated when free radicals abstract hydrogen from polyunsaturated fatty acids to form fatty acid radicals with a carbon- center, and then form lipid peroxyl radicals (Lipid-OO^-) via molecular rearrangement of lipid when they react with O_2 to form a conjugated diene.

$$\text{Lipid-H} + OH^- \rightarrow H_2O + \text{Lipid}^-$$

$$\text{Lipid}^- + O_2 \rightarrow \text{Lipid-}OO^-$$

These lipid peroxyl radicals are capable of reacting with other lipids, proteins, and nucleic acid and thereby cause subsequent oxidation of various biological molecules (Bagchi, M. *et al.* 1989; Kellogg, E.W. & Fridovich, I. 1975). Isoprostanes and hydroperoxides, are both formed by lipid peroxidation with

hydroperoxides, the major product (Radak, Z. 2000), being rapidly converted to a variety of more stable end-products including conjugated dienes, alkanes and aldehydic compounds such as malondialdehyde and 4-hydroxyalkenals (de Zwart, L.L. *et al.* 1999). Damage of macromolecules caused by reactive oxygen species and breakdown products of these molecules that are commonly measured are shown in Figure 6.1. Lipid oxidation can result in changes in membrane fluidity, structure and function (Beckman, K.B. & Ames, B.N. 1998). This contributes to the calcium overload in ischemia-reperfusion, and ultimately leads to the death of cardiomyocytes (Carini, R. *et al.* 1992). Lipid peroxidation of myocardial cell membranes has also been implicated as a potential cause of the ventricular tachycardia and fibrillation which are associated with ischemia reperfusion (Hearse, D.J. & Tosaki, A. 1987; Kloner, R.A. *et al.* 1989).

Antioxidant defence systems have evolved in living organisms to counteract the potential damage caused by the generation of reactive oxygen species. Antioxidants can be classified into two major categories: enzymatic, including superoxide dismutase, glutathione peroxidase and catalase, and non-enzymatic, including glutathione and dietary antioxidants such as vitamin E, vitamin C and beta-carotene.

Superoxide dismutase, which catalyzes the dismutation of the superoxide anion to hydrogen peroxide and oxygen, exists in three forms: manganese-containing superoxide dismutase, is found in mitochondria and copper and zinc containing superoxide dismutase, is found in the cytosol as well as on cell surfaces (Benzi, G.

1993). The third form, iron containing superoxide dismutase, is found only in bacteria (Radak, Z. 2000).

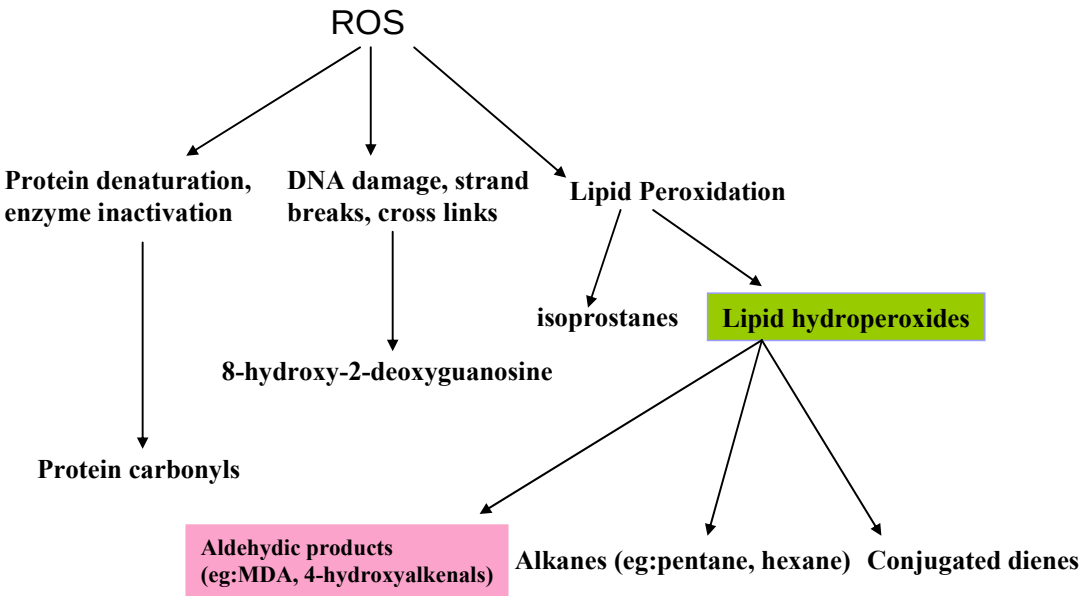


Figure 6.1 Damage of macromolecules caused by reactive oxygen species (ROS) and breakdown products of these molecules that are commonly measured.

Catalase and glutathione peroxidase catalyse the reduction of hydrogen peroxide to water, and glutathione peroxidase also converts hydroperoxides to alcohol (Powers, S.K. & Lennon, S.L. 1999).

Earlier in this thesis (Chapter Four), the analysis of membrane fatty acids in the heart revealed high incorporation of the long-chain (n-3) PUFA, DHA, (containing six double bonds). This produced an increase in peroxidization index of the membrane and suggested a higher oxidation risk with fish oil feeding. This increased oxidation potential predicts more oxidation products in myocardium,

which could lead to greater damage to myocardial cells. However, (n-3) PUFA are found to reduce or prevent ischemic damage, as illustrated in Chapter Five and other studies (Den Ruijter, H.M. *et al.* 2007; McLennan, P.L. *et al.* 1992a, 1993; McLennan, P.L. 2001; Pepe, S. & McLennan, P.L. 2002; Xiao, Y.-F. *et al.* 2008). Furthermore, there is no clinical evidence to suggest that fish oil supplementation or regular fish consumption promotes oxidative stress-related cardiovascular disease such as enhanced atherosclerosis. Although fish oil may increase oxidation risk, this may not be entirely detrimental. Accumulating evidence suggests that reactive oxygen species are not only injurious by-products of cellular metabolism but are also essential participants in cell-signaling and regulation (Rhee, S.G. 1999). The increase in lipid peroxidation products from fish-oil derived fatty acids may even cause adaptive responses that increase in-vivo antioxidant capacity. For example, prior dietary fish oil supplementation in the rat decreases the production of superoxide anion in myocardium undergoing ischemia and reperfusion (Supari, F. *et al.* 1995), suggesting reduced free-radical production and/or increased free radical scavenging.

Ischemic preconditioning has proved experimentally to be one of the most effective methods of protection against myocardial ischemic damage. Oxygen-derived free radicals, which are released during sublethal ischemia, are suggested to induce cellular defence mechanisms, which may act as triggers of ischemic preconditioning, protecting against further oxidative stress (Rudiger, H.A. *et al.* 2003). Furthermore, a growing body of evidence suggests that the administration of free radical scavengers prevents the postischemic benefits of ischemic -

preconditioning (Chen, W. *et al.* 1995; Forbes, R.A. *et al.* 2001; Tritto, I. *et al.* 1997). This all points to a cardioprotective role of free radicals. In parallel, it has been shown that diazoxide, a mitochondrial K_{ATP} channel opener, triggers the protective mechanism of ischemic preconditioning via free radical production (Baines, C.P. *et al.* 1997; Cleveland, J.C. *et al.* 1997). The administration of free radical scavengers, *N*-2-mercaptopropionyl glycine (Baines, C.P. *et al.* 1997; Cleveland, J.C. *et al.* 1997), or dimethylthiourea (Das, D.K. *et al.* 1999), abolish this protective effect of diazoxide in isolated rabbit and rat hearts. These data suggest that under some conditions, free radicals exert a paradoxical protective rather than deleterious effect in myocardium (Zhou, X. *et al.* 1996).

On the basis of these observations it was the aim of this study to test the hypotheses that,

- Increased peroxidization index of myocardial membrane will be associated with increased lipid oxidation products in the heart;
- The increase in oxidation products will be associated with increased endogenous antioxidant concentrations;
- Increased antioxidant concentrations at rest will be associated with reduced oxidation of lipids under the oxidative stress of ischemia and reperfusion and reduced cellular damage (infarct size)
- On this basis, the observed membrane changes with (n-3) PUFA feeding may be predicted to promote background lipid oxidation in the heart but

reduce the lipid oxidation associated with acute ischemia and reperfusion and reduce infarct size.

6.2 MATERIALS AND METHODS

This section describes the diet treatment of rats, and the steps to measure oxidative stress markers and antioxidants.

Diet treatment and heart perfusion

Rats were separately treated with the SF diet, (n-6) PUFA diet or (n-3) PUFA diet for six weeks as previously described. For baseline oxidative stress biomarkers and antioxidant measurements, left ventricular tissues were obtained from hearts which were subjected to 210 minutes perfusion without ischemia intervention **[normoxic perfusion]** (perfusion protocol see Chapter Three, Figure 3.4 and Chapter Five for example). For testing the effects of ischemia alone on oxidative stress biomarkers, antioxidants, and infarct size **[control experiments]**, the left ventricular ischemic regions and non-ischemic regions were obtained from hearts that were subjected to 30 minutes initial equilibration perfusion, 30 minutes control perfusion, 30 minutes regional ischemia and 120 minutes of reperfusion, a total of 210 minutes perfusion (see Chapter Three, Figure 3.4B and separation of ischemic and non- ischemic region was described in Chapter Five, page 112). For testing the effects of ischemia on oxidative stress biomarkers, antioxidants, and infarct size after ischemic preconditioning **[ischemic preconditioning experiments]**, the left ventricular ischemic region and non-ischemic region were obtained from hearts subjected

sequentially to 30 minutes to initial equilibration perfusion, 3*5- minute cycles of global ischemia and reperfusion prior to 30 minutes of regional ischemia and 120 minutes of reperfusion, for a total of 210 minutes perfusion (see Chapter Three, Figure 3.4C). At completion of the perfusion protocols, hearts were retained for either infarct size estimation or measurement of oxidation products and antioxidants. Samples of fresh tissue were used immediately for lipid hydroperoxide analysis and the remainder was rapidly frozen and stored at -80°C for analysis of other markers of oxidation status.

Measurement of oxidative stress biomarkers

Lipid hydroperoxides

Lipid hydroperoxides were measured using fresh tissue. Immediately on completion of each perfusion protocol, left ventricular samples from ischemic zone and non-ischemic zones were obtained from control hearts and ischemic preconditioned hearts. They were weighed and homogenized in 4mL of ice-cold pure water. A 0.5mL aliquot of homogenate was used for the lipid hydroperoxide assay while the rest was stored at -80°C for later analysis. The assay was conducted using Lipid Hydroperoxide Assay kit (Cayman Chemical Company, USA) and was performed in a 96-well glass plate using a Power Wave 340 spectrophotometer (BiO-TEK Instruments, Inc). A protein assay of the tissue homogenate was conducted as described below and lipid hydroperoxides were expressed per mg of heart protein.

Malondialdehyde (MDA) by HPLC

636-734-7032Frozen heart tissues were thawed on ice prior to analysis. To 500µl of tissue homogenate 1mL of 5% trichloroacetic acid and 700µl of 0.6% (w/v) thiobarbituric acid were added. After heating (45 minutes, 90°C), the samples were centrifuged (10 minutes, 4000 *g,* 4°C), and 120µl of sample injected onto the HPLC column in duplicate. The HPLC column used was reverse-phase C18 (SGE): 250 x 4.6mm stainless steel, 5um particle size. The resulting thiobarbituric acid-MDA complex was separated on reversed-phase HPLC and quantitated with a fluorescence detector (excitation 532 nm, flow rate 1.2mL/min) (Lepage, G. *et al.* 1991).

6.2.1 Protein Assay

Frozen heart tissues were thawed on ice prior to analysis. Protein assays were conducted using tissue homogenates and homogenate supernatant used in a variety of assays. 1.5 mL Coomassie Plus ^{TM}- The better Bradford Assay Reagent (Pierce, USA) was added to 0.05mL of sample and incubated at room temperature for 10 minutes. A calibration curve was produced by measuring the absorbance of increasing concentrations of bovine serum albumin (0-1.5mg/mL) at 595nm. The absorbance of each sample was then measured and the concentration was calculated from the standard curve. All samples were assayed in duplicate and the mean protein concentration was recorded. All protein assays were completed using a Shimadzu UV-1601 UV-visible Spectrophotometer (Seevaratnam, R. *et al.* 2009).

6.2.2 Measurement of endogenous antioxidant enzymes and α-tocopherol

Superoxide Dismutase Activity

Frozen heart tissues were thawed on ice prior to analysis. Total superoxide dismutase (SOD) activity and cytosolic copper zinc superoxide dismutase activity (CuZnSOD) were measured in normoxic heart tissue, ischemic heart tissue and, non-ischemic heart tissue using a BIOXYTECH®-SOD -525™ assay kit (Oxis Research ™, USA). Samples were homogenized in 4mL of pure water and spun at 3000rpm and 4°C for 10 minutes on a Hettich Zentrifugen Rotina 46R Centrifuge. Both total SOD and CuZnSOD activity were measured as per instructions provided in the assay kit and the activity of mitochondrial SOD activated by manganese (MnSOD), was calculated as the difference between total SOD and CuZnSOD. Absorbance was measured in a Cary 3000 Bio UV-Visible Spectrophotometer. All SOD activity is expressed per mg of protein of tissue homogenate supernatant.

Glutathione Peroxidase Activity

Frozen heart tissues were thawed on ice prior to analysis. Glutathione peroxidase (GPX) activity was measured in normoxic heart, ischemic and, non-ischemic heart tissue using BIOXYTECH®GPx-340TM colorimetric assay kit (OxisResearch ™,USA). Heart samples were homogenized in 8 volumes (per weight tissue, 1g=1mL) in cold buffer (50mM TRIS base, pH 7.5 containing 5mM EDTA,1mM 2-mercaptoethanol) before centrifugation at 5000rpm and 4°C for 30 minutes using an eppendorf centrifuge 5417R. The assay was then performed on the supernatant as described in the assay kit instructions. Absorbance was measured using a Cary 3000 Bio UV-Visible Spectrophotometer and is expressed as per mg of tissue

protein. A protein assay was evaluated on the diluted supernatant used for the assay.

Myocardial Alpha-tocopherol

Frozen heart tissues were thawed on ice prior to analysis. Myocardial alpha-tocopherol concentrations were measured by HPLC with electrochemical detection using a modification of the method previously described by Yang (Yang, C.S. & Lee, M.J. 1987). Approximately 200-300mg heart tissue was homogenised in 2mL of ice cold NaCI. Two hundred microliters of sample was placed into a tube, 175µl of ethanol and 25µl of an internal standard were added to the tube. The internal standards were obtained from Sigma-Aldrich –shown with corresponding catalogue number; all HPLC grade: alpha-tocopherol (T3251), delta-tocopherol (T2028), gamma-tocopherol (T1728), and alpha-tocopherol acetate (T3001). Standard cocktails were made up at concentrations of 2 ug/mL, 5 ug/mL, 10 ug/mL, 15 ug/mL and 20 ug/mL with a 10uL injection. A standard curve was included at the beginning, middle and end of a batch of samples on the HPLC. The HPLC technique was optimised for clear separation of these 4 standard peaks. This ensured the peaks in rat's samples were quantified correctly, ensuring no over-reporting due to interference from similar compounds. All samples from the same rat were run consecutively, in the same batch.

The sample solution was mixed with 400 µl of hexane, and vigorously shaken for two minutes. After centrifugation at 3000 rpm for 10 minutes, 300µl of hexane layer was taken up and evaporated. The residue was dissolved in 150 µl of the

mobile phase, and then was injected into HPLC system. Mobile phase was Acetonitrile/Methanol (60:40). All solvents were HPLC grade. Mobile phase was filtered and sonicated prior to use in the HPLC. Flow rate of 1.5 mL/min. Column – SGE 250 x 4.6mm stainless steel, reverse phase 5um particle size.

Determination of infarct size

The basic principle for determination of infarct size was similar to the experiment conducted by Liu *at al* (Liu, *Z. et al.* 2002). On completion of the ischemia-reperfusion protocol, the coronary artery was re-occluded and the heart was infused with Evans Blue dye to stain the non-ischemic tissue. Hearts were immediately placed in cold Krebs Henseleit, blotted dry and weighed. Hearts were then wrapped in a clear plastic food wrap and put in a freezer at -20°C for 40 minutes. The tissue can be cut with precision slice thickness when in a semi frozen state, as it puts the tissue into rigor and makes it easier to slice. Ventricles were sectioned into five transverse slices in a plane parallel to the atrioventricular groove. Heart slices were photographed for measurement of zone at risk and then incubated in a buffer containing triphenyltetrazolium chloride and sodium phosphate (pH 7.4) in a 37°C water bath for 15 minutes to reveal the infarct area. During the process, heart slices were gently agitated at least once a minute to make sure adequate exposure of each side of slices to triphenyltetrazolium chloride. Tripenyltetrazolium can cross the cell membrane and stains viable tissue in red. Once the colour has been established, the incubated slices were stored in 10% formalin for about 20 minutes to bleach the unstained tissue and increase the contrast. The living tissue was coloured brick red and the infarcted tissue was bleached free of colour. At this stage, heart slices were

photographed again and analysed for infarct size using the ImagePro program. In the initial photograph, the ischemic zone at risk of infarction was identified as the area of each slice that did not turn blue (i.e., not perfused with dye) after perfusion with the solution containing Evans blue dye. In the second photograph, the portion of the ischemic zone at risk of infarction that did not turn red in response to triphenyltetrazolium chloride incubation and remained white was classified as infarct area (dead tissue). The ischemic area at risk (unstained by Evans blue dye) and the infarcted area (unstained by triphenyl-tetrazolium chloride) were measured using ImagePro program software in trace-measurement mode, which is a sum of calibrated pixels in a defined region, through manually drawing an image layer on the photograph. The ischemic zone at risk of infarction and infarct area obtained from each side of a single slice, and the mean of both sides was used as the representative ischemic zone at risk of infarction and infarct area for that slice. The ischemic zone at risk of infarction or infarct size was determined by measuring the area of the ischemic zone at risk of infarction or infarction in a series of slices and then multiplying the area by the slice thickness of ~ 2mm. The volumes of each slice were summed to calculate the total volume of the ischemic zone at risk of infarction and infarct for each heart. Final infarct size was reported as a percentage of the zone at risk (Figure 6.2).

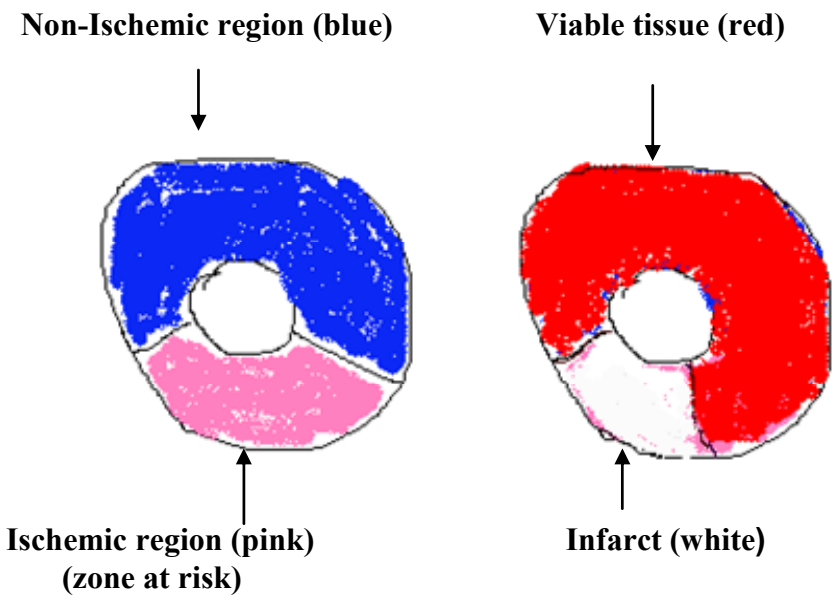


Figure 6.2 Illustrated pictures showing non-ischemic and ischemic region after injection of Evens blue dye and infarct size after triphenyl-tetrazolium chloride method.

6.2.3 Statistical analyses

Comparison was made between dietary groups and treatment groups with two-way analysis of variance (ANOVA) for diet and treatments (normoxic perfusion, IPC, control) main effect and diet x treatment interaction, and by multi-way ANOVA for diet, treatment and ischemic versus non-ischemic main effects with diet x ischaemia x treatment interaction. Tukey's test was used for post-hoc pairwise comparison of individual means and interactions. Results of significant interactions (such as illustrated in table 6.1A) are not specifically presented in graphs and tables but are presented as the results of further individual pairwise comparisons (as shown in table 6.1C). Linear regression was used to determine the relationship between measurements. Linear regression was used to determine

the relationship between measurements. All statistical analyses were performed using SPSS v.11.5 (SPSS Inc., Chicago, IL, USA) or Statistix (version seven) (Analytical software, Tallahassee, FL,USA) with significance accepted at $P<0.05$.

Example data analysis using lipid hydroperoxide measurement

The ANOVA was conducted for heart zones (ischemic and non-ischemic zones of the heart) and diet as main effects and analyzed for the interaction between diet and heart zones incorporating ischemic preconditioned and non-preconditioned (control) hearts for each diet. The detailed results of ANOVA and internal analysis for lipid hydroperoxide measures and subsequent display of results have been separated for baseline effect of diet excluding the effect of ischemia (Table 6.1B). Then, the effects of ischemia were evaluated excluding the normoxic hearts which do not have an ischemic region (Table 6.1C). Example analyses of lipid hydroperoxide are shown in Table 6.1(A-C).

Table 6.1A Analysis of Variance Table for LPO

Source	DF	SS	MS	F	P
Heart zones	4	1323.50	330.875	19.24	0.0000
Diet	2	138.60	69.302	4.03	0.0223
Heartz*diet	8	606.00	75.750	4.40	0.0003
Error	66	1135.26	17.201		
Total	80				

Heart zones: Isch_z, non-Isch, IPC-Isch-z, IPC-non-Isch_z, Normoxic. DF=4. Diet: SF, (n-6) PUFA, (n-3) PUFA, DF=2. Interaction heartz (zones)*diet, DF=8.

Table 6.1B Tukey HSD All-Pairwise Comparisons Test of LPO for diet

Diet	Mean	Homogeneous Groups
n-3	43.764	A
n-6	42.560	AB
SF	40.640	B

Alpha 0.05 Standard Error for Comparison VARIES

Table 6.1C Tukey HSD All-Pairwise Comparisons Test of LPO for hearta*diet

In control and IPC hearts

Heart zones	dieta	Mean	Homogeneous Groups
Isch_z	SF	54.417	A
Isch_z	n-6	49.400	AB
Isch_z	n-3	46.000	ABC
non-Isch	**n-3**	**45.500**	**BCD**
IPC-NonIsch_	**n-3**	**43.400**	**BCDE**
IPC-Isch_z	n-6	42.800	BCDEF
IPC-Isch_z	n-3	42.600	BCDEF
IPC-Isch_z	SF	41.100	BCDEF
non-Isc	**n-6**	**41.000**	**BCDEF**
IPC-NonIsch_	**n-6**	**40.000**	**CDEF**
non-Isch	**SF**	**37.000**	**DEF**
IPC-NonIsch_	**SF**	**34.167**	**F**

Table 6.1 Shows statistical analysis and example illustrating significant effect of heart zones (ischemic and non-ischemic), significant effect of diet and significant interaction between heart zones and diet. In table 6.1B and C, the ANOVA table is broken down for pairwise comparison of diet and heart and diet interaction showing where the significant differences occur between diets and between heart zones and diets.
Interactions sharing a common letter are not significantly different between diets, $p<0.05$. For example, ANOVA (Table 6.1A). shows significant effects of different heart zones (including ischemic preconditioned, control and normoxic hearts for all diets), $p< 0.0000$, significant effects of diet $p= 0.022$ and interaction $p= 0.0003$. Comparisons of effects of diet on LPO shows significant difference between (n-3) PUFA (greater) and SF diets (Table 6.1B). highest LPO production in SF diet ischemic zone was not significantly different to (n-6) PUFA or (n-3) PUFA ischemic zones for control hearts but was different to the SF non-ischemic zone of control hearts and both ischemic and non-ischemic zones for ischemic preconditioned SF hearts. By comparison, LPO measured in (n-3) PUFA ischemic zone was not significantly different to the (n-3) PUFA non-ischemic zone of control hearts or either zone in (n-3) PUFA ischemic preconditioned hearts.

6.3 RESULTS

This section describes effects of diet and preconditioning on oxidative stress and antioxidant capacity in the normoxic heart and the effects of diet and preconditioning on oxidative stress biomarkers and antioxidant capacity in hearts subject to ischemia.

Oxidative stress and antioxidant capacity in the non-ischemic myocardium

Lipid Hydroperoxide and Malondialdehyde

There were no significant differences in concentration of lipid hydroperoxide between the non-ischemic region of control hearts; non-ischemic region of the ischemic-preconditioned hearts; and the normoxic-perfused hearts, within any of the dietary groups (Figure 6.3). When these non-ischemic heart tissues were combined to compare the effects of diet, the concentration of lipid hydroperoxide was significantly greater in (n-3) PUFA than either (n-6) PUFA and SF hearts, ($P<0.05$) [LPO(nmol/mgprotein): SF 35.9±0.6 n=19; (n-6) PUFA 40.2±0.9 n=15; (n-3) PUFA 44.4±1.3 n=17)] (Figure 6.3A). Also, the concentration of lipid hydroperoxides was significantly greater in (n-6) PUFA than SF hearts ($p<0.05$).

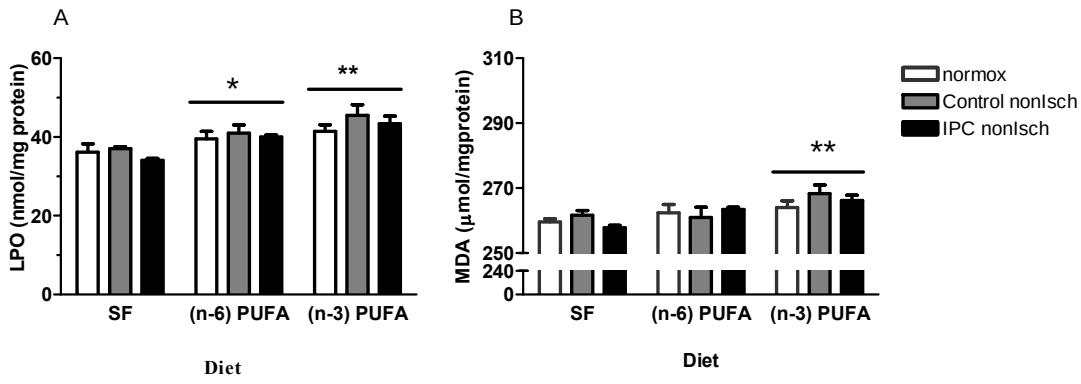


Figure 6.3 Influence of dietary supplements on A: LPO and B: MDA in nonischemic regions of rat isolated hearts. Data represents hearts that were normoxic throughout (normox) or the non-ischemic (nonIsch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Open bars: normoxic hearts, filled bars: control hearts. Lined bars: Ischemic preconditioned (IPC) hearts. *: Significant difference to SF diet groups ($p<0.05$) (ANOVA), **: Significant difference to both SF and (n-6) PUFA group ($p<0.05$) (ANOVA). Values represent mean ± SEM. n=5-7 per column.

Within the dietary groups, there were no significant differences in concentration of malondialdehyde between the non-ischemic tissues (Figure 6.3B). Comparing the dietary groups, the concentration of malondialdehyde was significantly greater in (n-3) PUFA than either (n-6) PUFA or SF hearts ($p<0.05$) [(MDA (µmol/mgprotein) SF 258.9±0.8 n=17; (n-6) PUFA 261.4±1.4 n=16; (n-3) PUFA 266.8±1.2 n=17)] (Figure 6.3B).

Superoxide Dismutase

There were no significant differences in concentration of total superoxide dismutase (Figure 6.4A) or manganese superoxide dismutase (Figure 6.4B) between the non-ischemic tissues within dietary groups. Between dietary groups, the concentration of total superoxide dismutase (Figure 6.4A) and manganese superoxide dismutase (Figure 6.4B) was significantly higher in (n-3) PUFA hearts

than in both (n-6) PUFA and SF hearts. Total SOD (units/mgprotein): SF 68.2±1.6 n=21; (n-6) PUFA 71.7±0.7 n=24; (n-3) PUFA 92.5 ±0.6 n=27)] ($p<0.05$). MnSOD (units/mgprotein): SF 52.9±0.9 n=21; (n-6) PUFA 54.8±0.7 n=24; (n-3) PUFA 75 ±0.6 n=27)]. There was no significant difference of total superoxide dismutase (Figure 6.4A) or manganese superoxide dismutase (Figure 6.4B) concentration between (n-6) PUFA and SF hearts in the non-ischemic hearts or heart regions. There was no significant difference in CuZnSOD concentration between (n-3) PUFA and (n-6) PUFA and SF hearts [CuZnSOD (units/mgprotein): SF 15.3±0.9 n=21; (n-6) PUFA 16.9±0.7 n=24; (n-3) PUFA 17.4±0.6 n=27)] ($p<0.05$) (Figure 6.5).

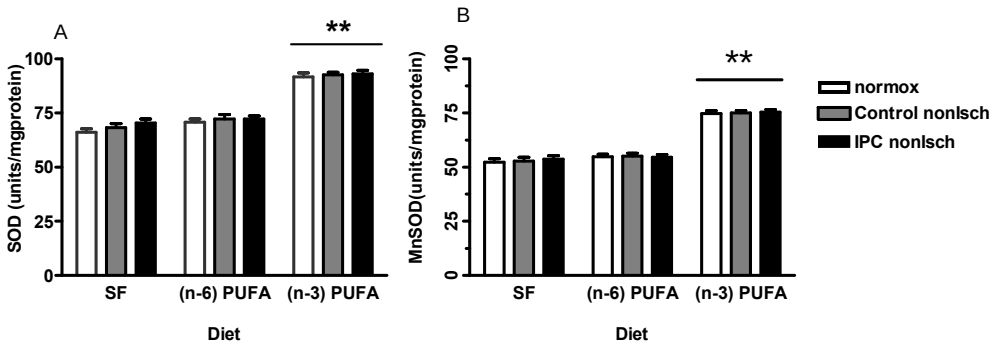


Figure 6.4 Influence of dietary supplements on A: total SOD and B: MnSOD in rat isolated hearts. Data represents hearts that were normoxic throughout (normox) or the non-ischemic (nonIsch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Open bars: normoxic hearts, filled bars: control hearts. Lined bars: Ischemic preconditioned (IPC) hearts. *: Significant difference to SF diet groups ($p<0.05$) (ANOVA), **: Significant difference to both SF and (n-6) PUFA group ($p<0.05$) (ANOVA). Values represent mean ± SEM. n=5-7 per column.

Glutathione Peroxidase

Within diets, there were no significant differences in Glutathione Peroxidase

concentrations for non-ischemic hearts or heart regions, nor were there any significant difference between diets. [GPX (mU/mgprotein): SF 19.2±1.5 n=21; (n-6) PUFA 19.7±1.5 n=24; (n-3) PUFA 21±1.2 n=27)] (Figure 6.6A).

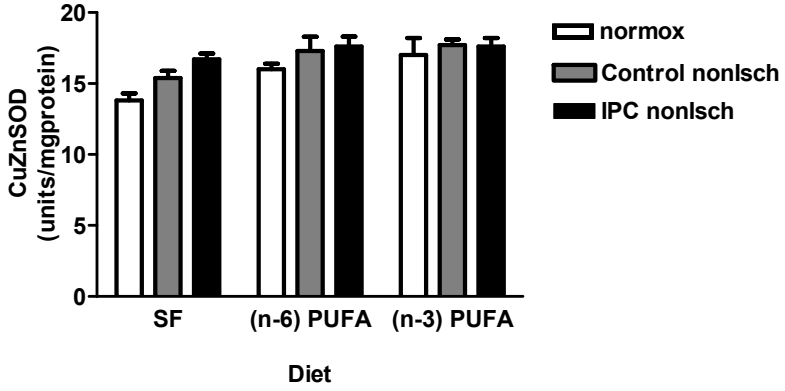


Figure 6.5 Influence of dietary supplements on CuZnSOD in non-ischemic regions of rat isolated hearts. Data represents hearts that were normoxic throughout (normox) or the non-ischemic (nonIsch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Open bars: normoxic hearts, filled bars: control hearts. Lined bars: Ischemic preconditioned (IPC) hearts. Values represent mean ± SEM. n=5-7 per column.

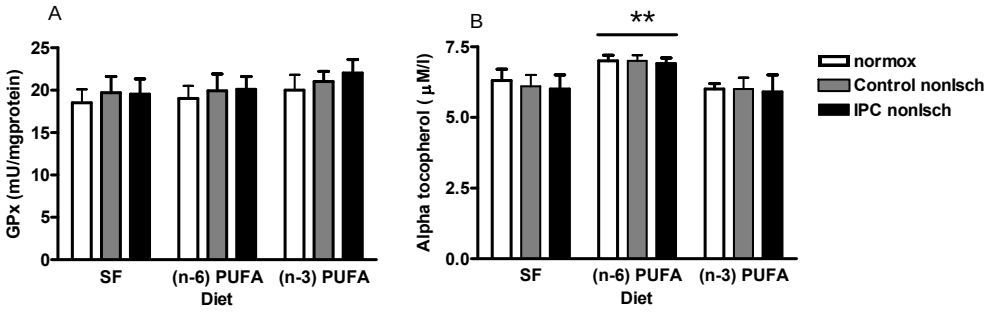


Figure 6.6 Influence of dietary supplements on A: glutathione peroxidase (GPx) and B: alpha tocopherol in rat isolated hearts. Data represents hearts that were normoxic throughout (normox) or the non-ischemic (nonIsch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Open bars: normoxic hearts, filled bars: control hearts. Lined bars: Ischemic preconditioned (IPC) hearts. *: Significant difference to SF diet groups ($p<0.05$) (ANOVA), **: Significant difference to both SF and (n-6) PUFA group ($p<0.05$) (ANOVA). Values represent mean ± SEM. n=5-7 per column.

Alpha tocopherol

The concentration of alpha tocopherol was significantly higher in (n-6) PUFA hearts than in both (n-3) PUFA and SF hearts ($p<0.05$) [alpha tocopherol (uM/L): SF 6.13±0.4 n=21; (n-6) PUFA 6.9±0.2 n=24; (n-3) PUFA 5.9 ±0.6 n=27)]. There was no significant difference in alpha tocopherol concentration between (n-3) PUFA and SF hearts (Figure 6.6B).

Oxidative stress biomarkers and antioxidant capacity in hearts subjected to ischemia

Lipid hydroperoxide

Within diets, there was no difference in LPO concentration between ischemic and nonischemic regions of (n-3) PUFA control hearts (Figure 6.7A). The LPO concentration was significantly greater in ischemic regions compared to the nonischemic regions in both (n-6) PUFA and SF hearts ($p<0.01$). Between diets, the concentration of LPO was lower in ischemic region of (n-3) PUFA control hearts compared to SF control hearts. There was no difference in LPO concentration in ischemic regions of control hearts between (n-6) PUFA and SF diets. With all dietary groups combined, LPO concentration was significantly lower in ischemic preconditioned hearts (ischemic plus nonischemic regions) than in control hearts (ischemic plus non-ischemic regions) ($p<0.01$) (Figure 6.7A). The concentration of LPO in ischemic regions of ischemic preconditioned hearts was not significantly different between diets (Figure 6.7A). With all dietary groups combined, the mean concentration of LPO was greater in ischemic regions than in non-ischemic heart regions ($p<0.01$).

Malondialdehyde

Within diets, there was no significant difference in MDA concentration between ischemic and nonischemic regions of (n-3) PUFA hearts (Figure 6.7B). The concentration of MDA was greater in the ischemic region than in the non-ischemic region of (n-6) PUFA control and SF control hearts ($p<0.01$). Between diets, the concentration of MDA was significantly lower in the ischemic region of (n-3) PUFA control hearts compared to SF control hearts (Figure 6.7B). There was no difference between (n-6) PUFA control hearts and SF control hearts (Figure 6.7B).

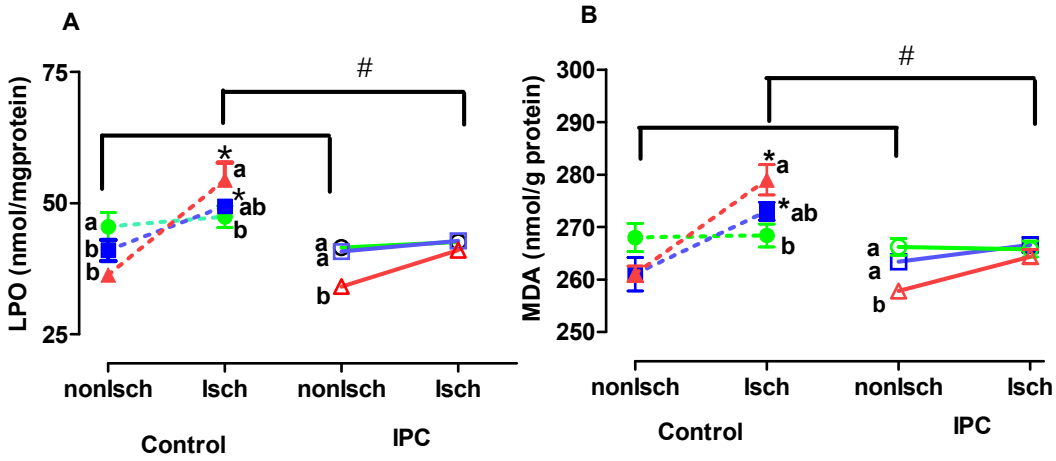


Figure 6.7A&B Influence of dietary supplements, ischemia and ischemic preconditioning on A: LPO and B: MDA in rat hearts. Data represents hearts that were non-ischemic (nonIsch) and ischemic (Isch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Non-filled symbols connected with solid line: Ischemic preconditioned groups, filled symbols connected with dotted line: Control groups. Δ triangle symbols: SF groups. □ Square symbols: (n-6) PUFA groups, ○ circle symbols (n-3) PUFA groups. *: Significant difference to its control group within the heart ($p<0.05$), Values sharing a common letter are not significantly different, vertically. Values represent mean ± SEM. n=6. # ischemia vs non-ischemia $p<0.01$.

With all dietary groups combined, MDA concentration was significantly lower in ischemic preconditioned hearts (ischemic plus nonischemic regions) than in control hearts (ischemic plus nonischemic regions) ($p<0.01$). The concentration of MDA in

ischemic regions of ischemic preconditioned hearts was not significantly different between diets (Figure 6.7B).

Superoxide Dismutase

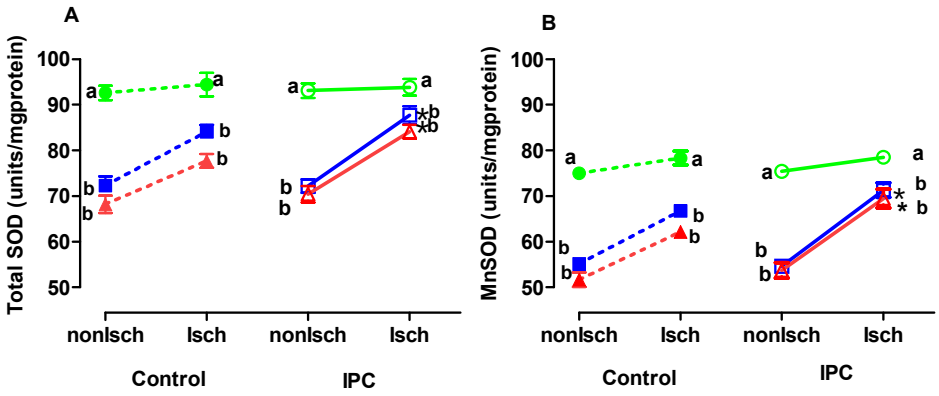


Figure 6.8 Influence of dietary supplements, ischemia and ischemic preconditioning on A: total SOD and B: MnSOD in rat hearts. Data represents non-ischemic (nonIsch) and ischemic (Isch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Non-filled symbols connected with solid line: Ischemic preconditioned groups, filled symbols connected with dotted line: Control groups. Δ triangle symbols: SF groups. □ Square symbols: (n-6) PUFA groups, ○ circle symbols: (n-3) PUFA groups. *: Significant difference to its control group within the heart ($p<0.05$), Values sharing a common letter are not significantly different, vertically. Values represent mean ± SEM. n=6

With all dietary groups combined, and control plus ischemic preconditioned hearts combined, the mean concentrations of total SOD (Figure 6.8A) and MnSOD were greater in ischemic regions than in non-ischemic heart regions ($p<0.01$) (Figure 6.8B). Between diets, the concentrations of total SOD and MnSOD in ischemic region of control hearts was higher in (n-3) PUFA hearts compared to (n-6) PUFA hearts and SF hearts. There was no difference in total SOD (Figure 6.8A) or MnSOD (Figure 6.8B) in ischemic region of control hearts between (n-6) PUFA hearts and SF hearts. Within diet, there were no significant differences in total SOD

and MnSOD concentration between ischemic and nonischemic regions of (n-3) PUFA control hearts (Figure 6.8A&B). The total SOD concentration and MnSOD concentration was greater in the ischemic region than the non-ischemic region in both (n-6) PUFA control and SF control hearts ($p<0.01$) (Figure 6.8A&B). With all dietary groups combined, total SOD concentration and MnSOD concentration were significantly higher in ischemic preconditioned hearts (ischemic plus nonischemic regions) than in control hearts (ischemic plus nonischemic regions) ($p<0.01$). Between diets, the concentration of total SOD and MnSOD was significantly higher in (n-3) PUFA hearts than (n-6) PUFA and SF hearts in ischemic regions of ischemic preconditioned hearts (Figure 6.8A&B). There were no significant differences in cytosolic (CuZn) SOD between diets or between control and preconditioned hearts (Figure 6.9).

The change between ischemic and non-ischemic regions (Δ) in LPO, MDA and MnSOD

The ischemic zone exhibited higher concentrations of lipid oxidation markers and antioxidants than the non-ischemic zone in control hearts (Figure 6.7& 6.8). After ischemic preconditioning, there were no differences in *Δ* LPO in the (n-3) PUFA and (n-6) PUFA hearts. The *Δ LPO* was significantly lower in SF ischemic preconditioned hearts compared to SF control hearts (Figure 6.10A).

There was no difference in *Δ* MDA concentration between preconditioned and control (n-3) PUFA hearts. The concentration of malondialdehyde was significantly

lower in ischemic preconditioned SF and (n-6) PUFA hearts than in their control (Figure 6.10B).

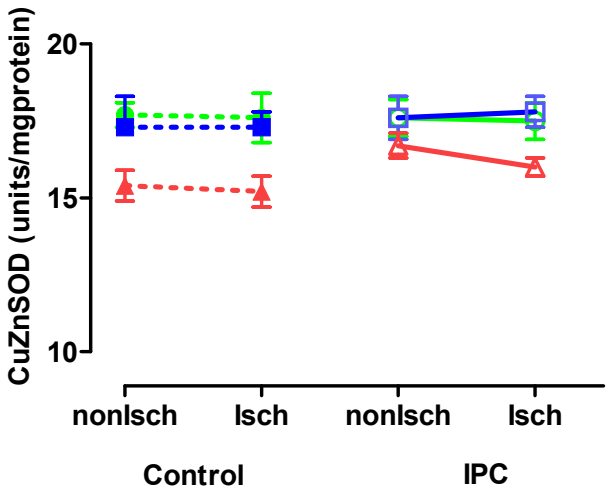


Figure 6.9 Influence of dietary supplements, Ischemia and Ischemic Preconditioning on cytosolic superoxide dismutase in rat hearts. Data represents non-ischemic (nonIsch) and ischemic (Isch) regions from control hearts or hearts that were subjected to 3*5min of ischemic preconditioning (IPC). Non-filled symbols connected with solid line: Ischemic preconditioned groups, filled symbols connected with dotted line: control groups. Δ triangle symbols: SF groups. □ Square symbols: (n-6) PUFA groups, ○ circle symbols: (n-3) PUFA groups. Values represent mean ± SEM. n=6

The *Δ* manganese superoxide dismutase was significantly lower in ischemic preconditioned (n-3) PUFA hearts compared to SF and (n-6) PUFA hearts (Figure 6.11).

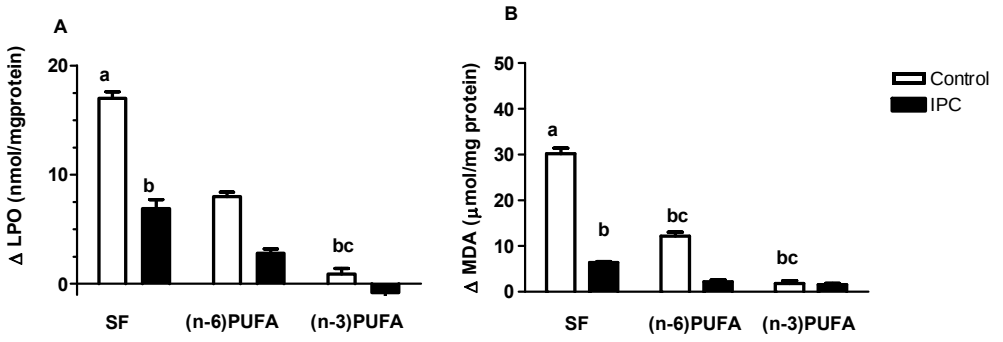


Figure 6.10 Effect of diet and ischemic preconditioning (IPC) on the difference in production of A: LPO and B: MDA in ischemic versus non ischemic regions (Δ) of the hearts. Non-filled symbols: The difference between, filled symbols: difference in IPC hearts. Diet groups without a common letter differ. Values represent mean ± SEM. n=6

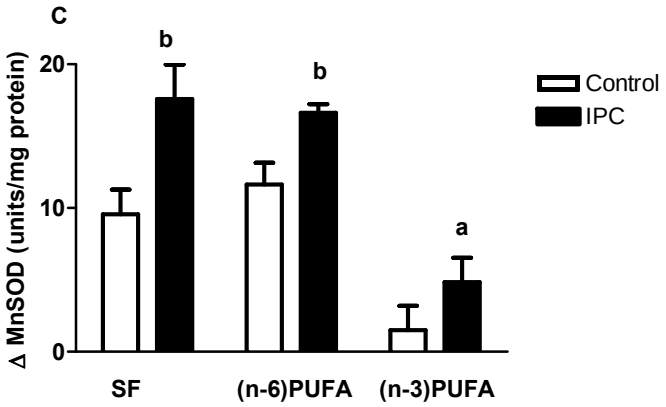


Figure 6.11 Effect of diet and ischemic preconditioning (IPC) on the difference in production of MnSOD in ischemic versus non ischemic regions of the hearts (Δ). Non-filled symbols: The difference between, filled symbols: difference in IPC hearts. Diet groups without a common letter differ. Values represent mean ± SEM. n=6

Effects of diet and preconditioning on infarct size

The infarct size as percent ischemic zone at risk was significantly smaller in control (n-3) PUFA hearts than in control SF or control (n-6) PUFA hearts ($P<0.05$) (Figure 6.12). There were no significant differences in infarct size between (n-6) PUFA and SF control hearts. There was no difference in the zone at risk between dietary groups

(n-3) PUFA: 0.26 ±0.02cm^3; SF: 0.28±0.02cm^3; (n-6) PUFA: 0.30±0.03cm^3), indicating that the degree of ischemia was equivalent. There were no significant differences in infarct size between preconditioned and control (n-3) PUFA hearts (P>0.05) (Figure 6.12). Ischemic-preconditioning significantly reduced infarct size in SF (P<0.05) and (n-6) PUFA hearts (P<0.05) representing an absolute decrease of approximately 35% of the zone at risk compared with control hearts.

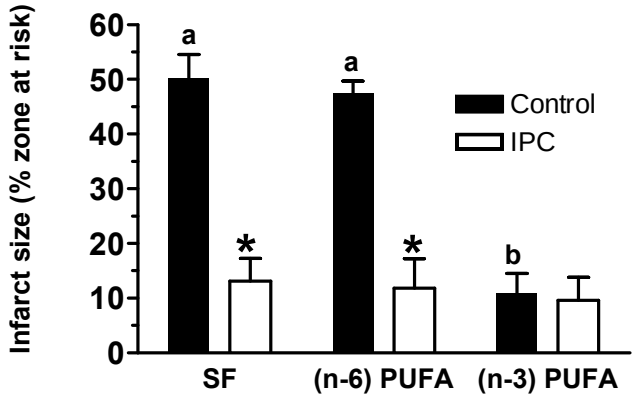


Figure 6.12 Influence of ischemic preconditioning on infarct size after 30 minutes ischemia and 120 minutes reperfusion in isolated perfused hearts from rats that consumed the SF, (n-6) PUFA, or (n-3) PUFA diets for six weeks. Values are means±SEM, n=6, * IPC and control groups differs within diet group, p<0.05. Within control or IPC groups, panels for a variable without a common letter differ between diets, p<0.01.

Association between antioxidants, oxidative damage and infarct size in control hearts

In control hearts, the MnSOD concentration was highly correlated (r^2=0.85) with lower infarct size resulting in a negative linear relationship between the concentration of MnSOD in non-ischemic region and infarct size, r^2=0.85 as shown in Figure 6.13.

A negative linear relationship between the concentration of MnSOD in non-ischemic region and the LPO and MDA concentrations in ischemic regions was also significant, with r^2=0.31 in MDA and r^2= 0.25 in LPO respectively. as shown in Figure 6.14. This suggested that MnSOD, present prior to ischemia prevented lipid peroxidation in ischemia- reperfusion.

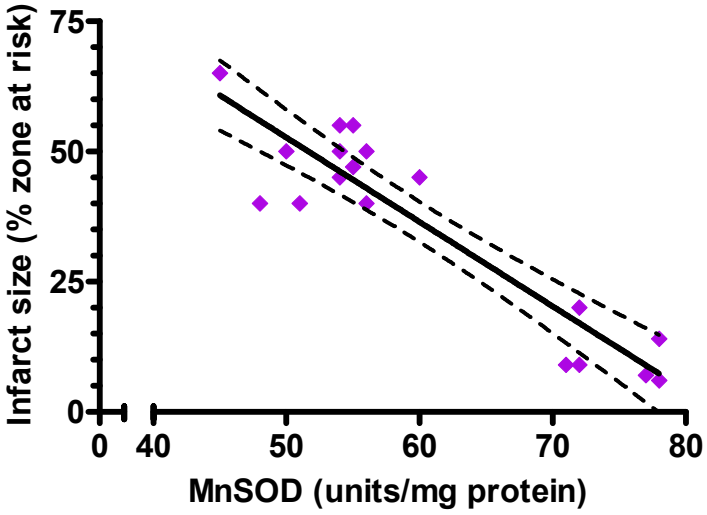


Figure 6.13 Scatter plot showing relationship between MnSOD in non-ischemic region of the heart on infarct size after 30 minutes ischemia and 120 minutes reperfusion in isolated perfused hearts from rats that consumed the SF, (n-6) PUFA, or (n-3) PUFA diets for six weeks. Values are means±SEM, n=6. r^2=0.851. Slope: p <0.001

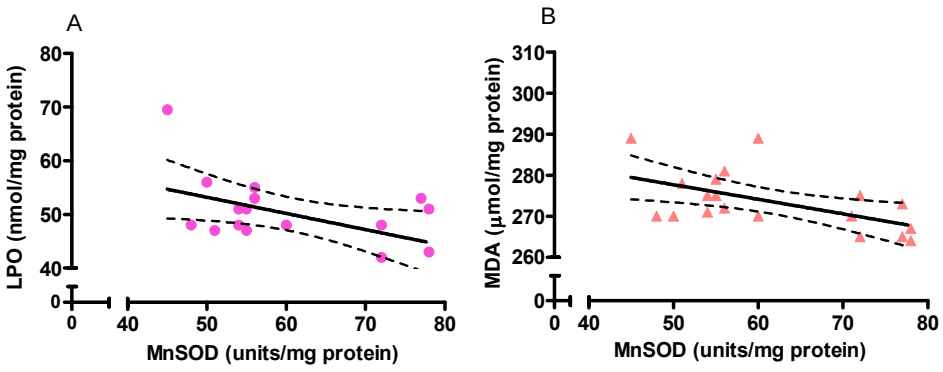


Figure 6.14 Scatter plot showing relationship between MnSOD in non-ischemic region of the control heart on A: MDA and B: LPO in ischemic region after 30 minutes ischemia and 120 minutes reperfusion in isolated perfused hearts from rats that consumed the SF, (n-6) PUFA, or (n-3) PUFA diets for six weeks. Values are means±SEM, n=6. MDA: r^2=0.31 LPO: r^2= 0.25. Slope: p <0.05.

Association between antioxidants, oxidative damage and infarct size in ischemic preconditioned hearts

In ischemic preconditioned hearts, there was no linear relationship between the concentration of MnSOD in the non-ischemic region and infarct size. However, the positive linear relationship between the concentration of MnSOD in non-ischemic region and MDA or LPO in the ischemic region was significant, with: r^2=0.41 in MDA and r^2= 0.36 in LPO respectively. ($p < 0.05$) as shown in Figure 6.15. The concentration of MDA and LPO were increased as MnSOD was increased. The ΔMnSOD in preconditioned hearts were correlated negatively with the concentration of MDA (r^2=0.31) and LPO (r^2=0.51) as shown in Figure 6.16.

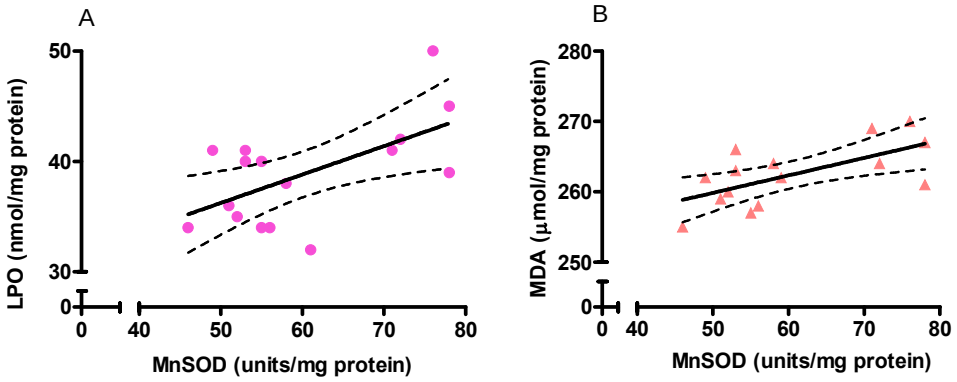


Figure 6.15 Scatter plot showing relationship between MnSOD in non-ischemic region and A: LPO and B: MDA in ischemic region after 30 minutes ischemia and 120 minutes reperfusion in isolated ischemic preconditioned hearts from rats that consumed the SF, (n-6) PUFA, or (n-3) PUFA diets for six weeks. Values are means±SEM, n=6. MDA: r^2=0.41. LPO: r^2=0.36. Slope: $p < 0.01$.

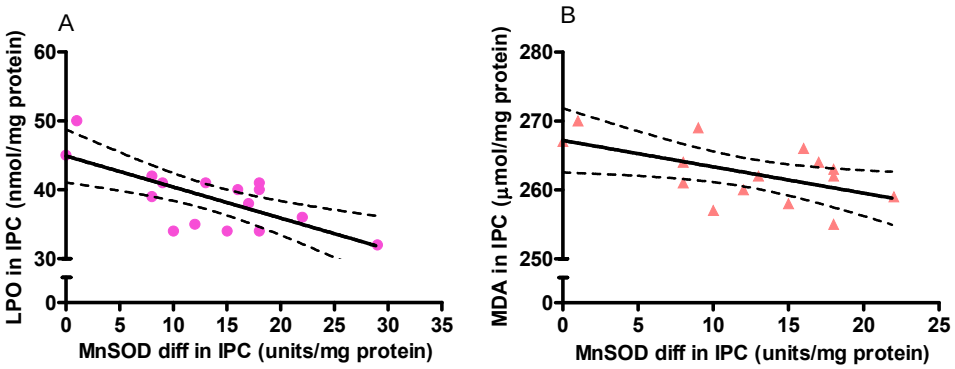


Figure 6.16 Scatter plot showing relationship between MnSOD in non-ischemic region of the heart and A: MDA and B: LPO in ischemic region after 30 minutes ischemia and 120 minutes reperfusion in isolated ischemic preconditioned hearts from rats that consumed the SF, (n-6) PUFA, or (n-3) PUFA diets for six weeks. Values are means±SEM, n=6. MDA: r^2=0.31. LPO: r^2=0.51. p <0.05.

6.4 DISCUSSION

This study has now directly verified the effect of (n-3) PUFA on limitation of infarct size, previously suggested from indirect evidence through the reduced release of cellular markers of damage in ischemia such as creatine kinase (Landmark, K. *et al.* 1998b; Pepe, S. & McLennan, P.L. 2002, 2007). Reduced infarct size is regarded as the ultimate indicator of cardioprotection by ischemic preconditioning (Liu, Y. & Downey, J.M. 1992). In the present study ischemic preconditioning was confirmed to significantly reduce infarct size after ischemia-reperfusion in SF and (n-6) PUFA hearts. However, while infarction was equally inhibited by ischemic preconditioning or fish oil feeding, the infarct size was not further reduced by the concurrent imposition of ischemic preconditioning with (n-3) PUFA prefeeding. Fish oil feeding therefore appears to produce “dietary” or

"nutritional" preconditioning equivalent to ischemic preconditioning in terms of infarct prevention.

To evaluate the involvement of MnSOD on limitation of infarct size, correlations were made between the concentration of MnSOD in non ischemic region (equal to normal hearts) and infarct size, in both control (all diets combined) and preconditioned (all preconditioned groups combined) hearts. A significant negative correlation was found between the concentration of MnSOD and infarct size in control hearts, suggesting a protective effect of endogenous antioxidants. No correlation was found between MnSOD and infarct size in preconditioned hearts, perhaps suggesting that the reduction in infarct size by ischemic preconditioning is not attributable to an acute increase in MnSOD concentrations. However, the difference in MnSOD between the ischemic and non-ischemic regions in ischemic preconditioned hearts was negatively correlated with the oxidation markers produced in ischemia. In turn, the markers of lipid oxidation were markedly lower in the ischemic zone of ischemic preconditioned hearts, as was the extent of infarction. This suggests that ischemic preconditioning increases MnSOD but only in conjunction with the major ischemic insult.

During ischemia or ischemia reperfusion, a gradual decrease in mitochondrial respiration occurs (Lesnefsky, E.J. *et al.* 1997; Vanden Hoek, T.L. *et al.* 1998). An immediate consequence of such decrease in respiratory function is the increase in the production of reactive oxygen species in the mitochondria (Werns, S.W. & Lucchesi, B.R. 1990). At high concentrations, the reactive oxygen species can induce lipid peroxidation of membranes, changing their integrity and increasing

their fluidity and permeability. Due to the short life and high reactivity of reactive oxygen species, it is difficult to measure them directly; however, it is possible to measure the products of reactive oxygen species that are more stable and an indicator of cellular damage. Lipid peroxidation is the marker of biological oxidation and MDA is an end product derived from peroxidation of polyunsaturated fatty acids and esters, measurement of these aldehydes provides an index of lipid peroxidation.

In this study, the (n-3) PUFA hearts had higher basal concentrations of the markers of lipid oxidation, LPO and MDA, as predicted from the membrane fatty acid peroxidation index in Chapter Four. However, an increase in lipid oxidation, induced by ischemia and reperfusion, was only observed in SF and (n-6) PUFA hearts. Thus, less lipid peroxidation occurred in (n-3) PUFA hearts in response to the acute oxidative stress. From the correlation analysis, it might be inferred that the increase in MnSOD induced by prolonged feeding of the (n-3) PUFA diet prevented the ischemia-induced increase in lipid oxidation. While, the ischemia-induced increase in lipid oxidation was also inhibited by ischemic preconditioning, the response was more complex, with the increase in MnSOD only apparent in this study when combined with the major ischemic insult (Das, D.K. *et al.* 1993).

Few studies have used lipid hydroperoxides as markers of lipid peroxidation in relation to fish oil consumption and the results have been inconsistent. It is well known that (n-3) PUFA are highly susceptible to *in vivo* oxidation, primarily auto-oxidation as they have multiple double bonds which are located deep in the mitochondrial membrane bilayer where most reactive oxygen species are produced

(Cho, S.Y. *et al.* 1978). Some studies of the rat heart (Ando, K. *et al.* 2000; Skuladottir, G.V. *et al.* 1994), kidney, lung or liver reported that the concentration of oxidative products is not affected by fish oil consumption (Ando, K. *et al.* 2000). On the contrary and in agreement with the finding of present study, others report increases in oxidative products associated with fish oil consumption (Song, J.H. *et al.* 2000; Song, J.H. & Miyazawa, T. 2001). This discrepancy may be ascribed to the use of high polyunsaturated (n-6) PUFA control diets. in those studies showing no differences (Ando, K. *et al.* 2000; Skuladottir, G.V. *et al.* 1994). As a polyunsaturated diet, (n-6) PUFA may also cause an increase in lipid peroxidation index if the concentration is high in heart tissues (Charnock, J.S. *et al.* 1983).

Fikova and co-workers observed increases in peroxidation risk with a very high fat (n-6) PUFA diet in which 37% of diet by weight was (n-6) PUFA sunflower seed oil with a double bond index was not significantly different to the high double bond index induced by an (n-3) PUFA diet (Fickova, M. *et al.* 1998). Charnock and co-workers (Charnock, J.S. *et al.* 1983) showed elevated double bond index with 12% sunflower seed oil supplement. The present study, which used only a 5% sunflower seed oil supplementation, did not elevate the unsaturation of the membrane compared to the SF diet and consequently failed to show an increase in oxidative products in the normoxic heart.

In normal physiological conditions, the production of reactive oxygen species is generally homeostatically controlled by endogenous free radical scavengers such as SOD, catalase, and GPX system, to protect the cell against cytotoxic oxygen metabolites. The SOD is an enzyme, which is primarily responsible for clearance of

superoxide anions. It catalyzes the dismutation of superoxide anion to hydrogen peroxide. Free radicals, while causing damage to cardiac myocytes, may also act as triggers of self-protecting mechanisms as discussed above.

In this study, (n-3) PUFA hearts had higher concentrations of superoxide dismutase in normoxic tissue (all the changes in total superoxide dismutase were attributable to mitochondrial derived MnSOD but not cytoplasm derived CuZnSOD). However, the modest increase in total SOD activity after ischemic preconditioning appeared in the (n-6) PUFA and SF hearts but not in the (n-3) PUFA hearts, in which it was already elevated. A moderate increase in oxidative products by (n-3) PUFA seems to stimulate an increase in SOD activity, which is at least equal to and possibly exceeds the enhancement in SOD activity during ischemia-reperfusion that is seen with ischemic preconditioning. This suggests that (n-3) PUFA have a preconditioning-like effect in up-regulating SOD activity.

The results of this study are in line with previous studies, which suggested that (n-3) PUFA may reduce oxidative stress by increasing SOD activities. Fish oil feeding produces increased renal (Chandrasekar, B. & Fernandes, G. 1994) and hepatic (Venkatraman, J.T. *et al.* 1994) expression of the antioxidant enzymes catalase and SOD in mice. Dietary supplementation with (n-3) PUFA enhanced resistance to free radical attack and reduced lipid peroxidation by inducing increased SOD activity in rats (Erdogan, H. *et al.* 2004). Barbosa and co-workers reported that the oxidative radicals were increased in plasma of patients with ulcerative colitis and there was a significant decrease when patients used (n-3) PUFA. They suggested that (n-3) PUFA may act as free radical scavengers, paradoxically protecting the

patients against the overall effect of oxidative stress (Barbosa, D.S. *et al.* 2003). Collectively, these reports support the results of present study, showing that moderate oxidation of (n-3) PUFA may induce resistance to more extreme oxidative stress by earlier stimulating SOD activity. A postulated scheme to explain the apparent paradox is illustrated in Figure 6.17.

In the present study, ischemic preconditioning followed by 30 minutes of ischemia and 120 minutes of reperfusion resulted in a modest increase in concentration of

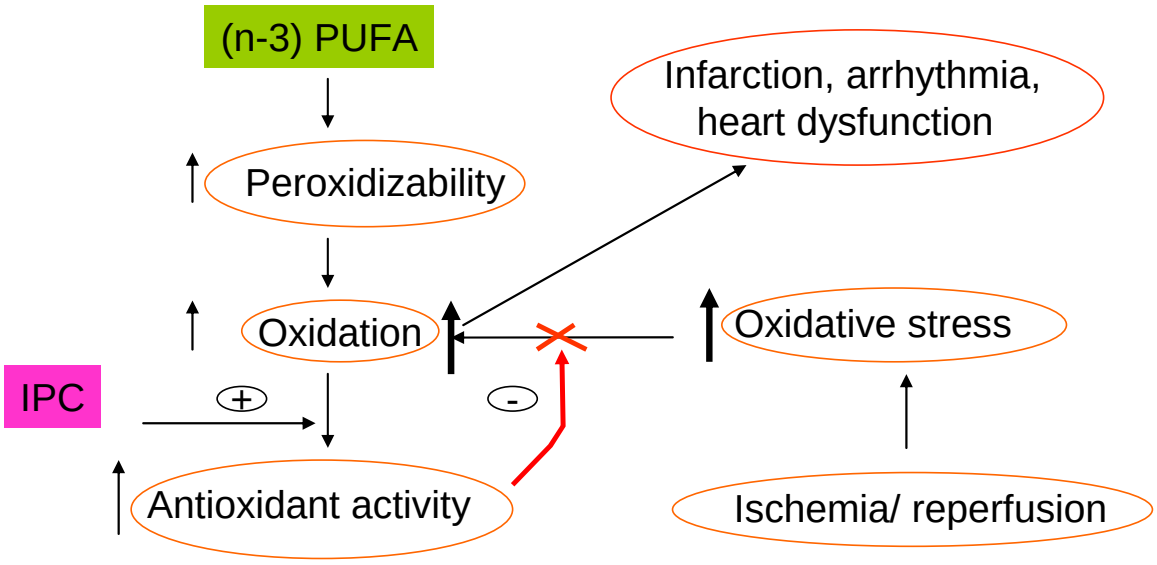


Figure 6.17 Diagram illustrating how (n-3) PUFA and IPC may reduce oxidative stress in response to acute oxidative stress

MnSOD in SF and (n-6) PUFA hearts. Ischemic preconditioning has two phases, an early phase and a late phase. The early phase of ischemic preconditioning occurs within minutes and disappears within two to four hours after the preconditioning stimulus. The late phase of preconditioning becomes manifest 24 hours after the ischemic preconditioning stimulus and lasts for 72 hours (Qiu, Y. *et al.* 1997). This

delayed ischemic preconditioning is closely related to the production of reactive oxygen species (Chen, W. *et al.* 1995; Hoshida, S. *et al.* 1993; Sun, J.-Z. *et al.* 1996; Zhou, X. *et al.* 1996) and the synthesis of reactive proteins after an initial ischemic burst (Rizvi, A. *et al.* 1999). This more clearly aligns (n-3) PUFA " nutritional preconditioning" with late ischemic preconditioning. However, some studies also report the involvement of MnSOD in the early phase of ischemic preconditioning (Das, D.K. *et al.* 1993; Zimmermann, R. *et al.* 1995) and a small increase in MnSOD was observed in the ischemic region in heart subjected to ischemic preconditioning (early phase) in this study. In line with present study an increased expression of genes for the antioxidant MnSOD induced by ischemic preconditioning was correlated with cardioprotection as evidenced by improved ventricular function and reduced infarct size (Das, D.K. *et al.* 1993).

It is suggested that the stimulation of antioxidant enzyme activities may reflect the heart's response, enabling it to survive against the ischemic stress by eliminating the oxidative assault (Zhou, X. *et al.* 1996). The preconditioning produced by the opening of potassium channels in mitochondria with diazoxide is also associated with increased oxygen-derived free radical production from the mitochondria (Pain, T. *et al.* 2000). Similarly, the involvement of oxygen-derived free radicals in ischemia reperfusion-induced damage was demonstrated in dogs, when acute intravenous administration of the free radical scavengers, superoxide dismutase and catalase, prevented the reduction in infarct size caused by ischemic preconditioning (Richard, V.J. *et al.* 1988). Ischemic preconditioning stimuli in canine hearts also resulted in an increase in MnSOD activity in the preconditioned myocardium,

whereas activities of other antioxidant enzymes such as GPX and CuZnSOD were not significantly changed (Hoshida, S. *et al.* 1993). Furthermore, it was reported that mice over expressing MnSOD are less sensitive to the damaging effects of ischemia/reperfusion (Chen, Z. *et al.* 1998). These data imply that MnSOD plays an important role in the protective effect of ischemic preconditioning as it does with that of the (n-3) PUFA.

The protective effects mediated through MnSOD localizes these effects to the mitochondria (Benzi, G. 1993). The mechanism by which mitochondrial oxygen-derived free radicals mediate the protective effect of preconditioning against ischemia is not yet fully known. It was suggested that free radicals could act as a second messenger to activate G-proteins (Bhatnagar, A. *et al.* 1990), protein kinases (Tokube, K. *et al.* 1996), and ATP-dependent potassium channels (Bhatnagar, A. *et al.* 1990; Thannickal, V.J. & Fanburg, B.L. 2000; Tokube, K. *et al.* 1996) in the ischemic preconditioning cascade. The increased concentration of superoxide dismutase converts superoxide to hydrogen peroxide, with increased MnSOD activity causing higher, yet non-lethal levels of peroxide. Part of this effect may be due to upregulated antioxidant mechanisms.

Elevated activity of GPX has been shown in liver, following fish oil consumption in the rat (Ruiz-Gutierrez, V. *et al.* 1999; Ruiz-Gutierrez, V. *et al.* 2001), but effects of dietary fish oil on GPX in the heart are not widely reported. In line with the study conducted by Ibrahim and his colleagues (Ibrahim, W. *et al.* 1999), there were no effects of diet on myocardial GPX activity in the present study. It was explained that the differences of concentration of GPX are due to the duration of

(n-3) PUFA feeding, four weeks compared to 12-15 weeks (Ibrahim, W. *et al.* 1999) . This explanation is relevant to present study where 6-week prefeeding with fish oil diet was not effective in causing any changes in GPX. The present study also found no effects of (n-3) PUFA on concentrations of the exogenous lipid soluble antioxidant α-tocopherol, however, there was a higher concentration of α-tocopherol concentration in (n-6) PUFA hearts. This may explain the effect of (n-6) PUFA in reducing the difference in LPO and MDA concentration in the ischemic region compared to SF hearts. Sunflower seed oil, like many plant oils, is a naturally rich source of α-tocopherol (USDA 2002). It has previously been reported that α-tocopherol concentration is decreased in both blood serum and liver in rats that have been fed a high fish oil diet, associated with elevation of lipid peroxidation products in the liver (Mouri, K. *et al.* 1984). Interestingly, Chautan and co-workers postulated that (n-3) PUFA consumption might cause redistribution of α-tocopherol within an organism when it was demonstrated that (n-3) PUFA causes a decrease in α-tocopherol concentration in the blood and the liver, while alpha-tocopherol was accumulated in the heart (Chautan, M. *et al.* 1990). Since, α-tocopherol concentrations were only measured in the myocardium in present study, the postulation about redistribution of α-tocopherol in different organs can not be assessed.

In summary, as hypothesised, the increase in peroxidation index identified in myocardial membranes of (n-3) PUFA hearts was associated with increased concentration of lipid hydroperoxide and malondialdehyde. In turn, this increased concentration of lipid hydroperoxide and malondialdehyde was then associated with increased concentration of endogenous antioxidant. The increased

concentration of antioxidant was associated with reduced oxidative response to acute ischemic stress.

The upregulation of superoxide dismutase in (n-3) PUFA and ischemic preconditioning in the present study suggests that (n-3) PUFA have some commonality with ischemic preconditioning. It seems that lipid peroxidation products and superoxide dismutase are involved in (n-3) PUFA-induced protection against ischemic damage, but glutathione peroxidase and the major *in vivo* lipid soluble antioxidant, α-tocopherol are not involved. Although the results of present study suggested that free radicals induced by (n-3) PUFA might possibly constitute one of the triggers of nutritional preconditioning, a close cause-effect relationship between free radicals, antioxidants activation and myocardial protection deserves further investigation. The increase in antioxidants may have inhibited the acute rise in oxidative products in ischemic preconditioned hearts and (n-3) PUFA treated hearts. However, since the incorporation of (n-3) PUFA and the increase in manganese superoxide dismutase is gradual, a dose response study of oxidation and antioxidant levels with higher or lower levels of (n-3) PUFA should be carried out. Measurement of oxidation and antioxidant products should also be made at various time points after ischemic preconditioning stimuli and during early stages of reperfusion, in order to more closely follow the cause and effect of oxidation, antioxidant activation and prevention of oxidation. In particular, the lag-time of membrane lipid oxidation may be altered (Dotan, Y. *et al.* 2004). Lag-time is a measure of resistance to peroxidation (commonly used as an indicator of atherosclerotic potential of LDL cholesterol), that can not be determined in the current study with only a single time point for evaluation of oxidation markers.

CHAPTER 7

GENERAL DISCUSSION

The present thesis demonstrates that dietary fish oil protects the heart against ischemic injury when hearts are subjected to occlusion of a major coronary artery, in a manner simulating an acute heart attack. The cardioprotection afforded by (n-3) PUFA and manifested as: reduction in infarct size, cardiac arrhythmias, and heart rate, all established factors contributing to risk of cardiovascular mortality (Herlitz, J. *et al.* 1994; Huikuri, H.V. *et al.* 2001; Kristal-Boneh, E. *et al.* 2000). Reduced ventricular end-diastolic pressure, especially under the influence of ischemia, increases in rate of relaxation, improvements in developed pressure and slowed heart rate, all suggest improved cardiac function (Gao, W.D. *et al.* 1995). These cardioprotective effects were demonstrable in comparison to both SF and (n-6) PUFA diets with the hearts from (n-6) PUFA fed rats not performing significantly differently to the saturated fat hearts in most measures. This indicates that the effects of fish observed in this thesis are specifically attributable to the (n-3) PUFA and not non-specific effects of lowered saturated fat or elevated polyunsaturated fat in the diet. The cardioprotection was associated with cellular incorporation of (n-3) PUFA into myocardial membranes.

This study has confirmed antiarrhythmic effects of (n-3) PUFA that have been reported in other studies summarised in (Matthan, N.R. *et al.* 2005). It directly verified (n-3) PUFA limitation of lethal myocellular injury and infarct size, which was

previously suggested from indirect evidence through the reduced release of cellular markers of damage in ischemia, such as creatine kinase and elevated serum potassium (Landmark, K. *et al.* 1998b; Pepe, S. & McLennan, P.L. 2002, 2007). Reduced infarct size is regarded as the gold-standard for demonstrating the cardio protective effects of ischemic preconditioning (Baxter, G.F. *et al.* 1997).

Although it has been previously suggested that (n-3) PUFA may act through changes in blood and platelet properties (Zhu, B.Q. *et al.* 1994), the *in vitro* nature of the present studies isolate these effects to intrinsic properties of the heart following membrane composition change. This also excludes influences on automatic nervous function or peripheral vascular effects that could contribute to reduced heart rate and arrhythmia *in vivo*. This conclusion is reinforced by lower heart rate with fish supplementation in human heart transplant patients (Harris, W.S. *et al.* 2006b) as well as with intact nervous control (Mozaffarian, D. *et al.* 2008). Indeed, the fatty acid composition of the hearts demonstrated, that in contrast to minor differences in membranes between hearts of rats fed saturated fat or (n-6) PUFA rich diets despite major dietary differences, feeding rats with fish oil caused major alterations in the membranes. The main influence was incorporation of DHA into heart membrane phospholipid when the diet had been supplemented with fish oil. This increased incorporation of DHA into the heart membrane was also associated with decreased concentrations of linoleic acid and arachidonic acid, which are the precursors of powerful mediators of inflammation, such as thromboxane, prostaglandins and leukotrienes (Calder, P.C. 2006).

Studies have shown that increases in the presence of DHA in tissue such as liver and kidney or in plasma increase susceptibility to peroxidation (Song, J.H. et al. 2000; Song, J.H. & Miyazawa, T. 2001). The observation of high unsaturation index and peroxidization index in (n-3) PUFA hearts was suggestive of a similar greater risk of oxidative damage. The (n-3) PUFA hearts did have higher basal concentrations of the markers of lipid oxidation, LPO and MDA, as predicted from the membrane fatty acid peroxidization index but without evidence of enhanced damage. Despite greater peroxidation potential and evidence of higher basal lipid oxidation with fish oil feeding, the acute increase in lipid oxidation induced by ischemia and reperfusion was observed only in SF and (n-6) PUFA hearts. Thus, less lipid peroxidation occurred in (n-3) PUFA hearts in response to the acute, damaging oxidative stress. By way of explanation, the elevated peroxidization index was also associated with a higher concentration of endogenous antioxidant MnSOD in (n-3) PUFA hearts. It seems that a moderate increase in oxidation products induced by (n-3) PUFA, that is in itself insufficient to cause damage, stimulates an increase in antioxidant activity. In turn, the protection against oxidation during and after ischemic stress was associated with reduced arrhythmias and improved functional recovery and ultimately, less myocardial damage in the form of infarction.

The present study suggests that reactive oxygen species and superoxide dismutase are involved in both (n-3) PUFA and ischemic preconditioning-induced protection against ischemic damage. Induction of intrinsic cardioprotective proteins such as MnSOD has been implicated in the signaling pathway of late ischemic preconditioning. Therefore, it is speculated that (n-3) PUFA may protect the heart

by activating a signaling pathway that in part resembles late ischemic preconditioning (Baxter, G.F. & Ferdinandy, P. 2001). Late preconditioning depends upon nuclear signaling and protein production (such as MnSOD). Similarly, exercise training was suggested as a form of preconditioning (Domenech, R. *et al.* 2002; Hamilton, K.L. *et al.* 2001). Rats subjected to 30 minutes of exercise exhibit increased MnSOD activity 48h after the exercise and this is associated with protection of the heart against ischemic damage (a delayed preconditioning) (Hamilton, K.L. *et al.* 2001).

Sublethal oxidative stress evoked by generation of ROS after sublethal ischemia is essential to trigger the delayed preconditioning (Sun, J.-Z. *et al.* 1996; Zhou, X. *et al.* 1996). Reactive oxygen species induce *denovo* synthesis of proteins (Pinkus, R. *et al.* 1996). Ischemic preconditioning and exercise also induce various proteins in heart tissue, including SOD (Das, D.K. *et al.* 1995; Oberley, L.W. *et al.* 1987; Powers, S.K. *et al.* 1993). The peroxidization index of myocardial membrane predicted an increase in oxidation that was confirmed by direct measures of lipid oxidation products. Therefore, reactive oxygen species produced from (n-3) PUFA may, as in ischemic preconditioning or exercise, augment the tolerance of heart to ischemia-reperfusion injury by inducing intrinsic rescue proteins such as MnSOD.

Because the consumption of fish or fish oil leads to sustained, elevated incorporation of DHA into myocardial membrane phospholipids (Owen, A.J. *et al.* 2004), DHA becomes continuously elevated by fish oil feeding. Therefore, the lipid oxidation observed here under basal perfusion conditions is likely to be continually

present, inducing a chronic elevation of MnSOD. The evidence of cardioprotection persist for periods of feeding beyond the six weeks in the present study to longer periods ranging from four to 30 months (McLennan, P.L. *et al.* 1988; McLennan, P.L. *et al.* 1992b; Pepe, S. & McLennan, P.L. 2002). With regular fish or fish oil consumption, the (n-3) PUFA are present at all times, prior to any unexpected ischemic episode. Therefore, the cardioprotective effects of fish or fish oil as a regular part of the diet, that reduce the consequences of ischemic events in the human population (Landmark, K. 1998), may be explained as a "nutritional preconditioning" effect of (n-3) PUFA. Unlike pharmacological mimicking of ischemic preconditioning, nutritional preconditioning would require no prediction or prior knowledge of ischemic events and could thus represent a low-risk solution to ischemic cardioprotection.

The (n-3) PUFA and ischemic preconditioning have both been shown to have effects on calcium influx, efflux or intracellular calcium redistribution in myocardial cells. The (n-3) PUFA modulate a variety of sub-cellular enzymes and ion channels, involved in intracellular calcium handling, in a way that individually or collectively may underpin the cardioprotection. Many of the published effects of (n-3) PUFA and observations from these studies such as slowed heart rate, prevention of arrhythmia, and improved relaxation can be related to influences on key enzymes involved in calcium handling. Intracellular calcium overload causes the generation of arrhythmias in isolated cardiomyocytes. The (n-3) PUFA prevent these arrhythmias via direct interaction with calcium regulatory enzymes such as L-type voltage-gated calcium channels (Leaf, A. *et al.* 1999; Leaf, A. 2001), low-voltage gated T-type calcium

channels (Danthi, S.J. *et al.* 2005), ryanodine receptor calcium release channel-II, calcium dependent calmodulin kinase II channel (Zaloga, G.P. *et al.* 2006), Na^{+}-Ca^{2+} exchange and Na-K^{+}ATPase pump (Verkerk, A.O. *et al.* 2006) (Figure 5.7). The (n-3) PUFA also alter phospholipase activities such as PLC and PLA_2, which play important roles in calcium mobilization (Grynberg, A. *et al.* 1992; Salem, N. *et al.* 1988) and subsequently change affinities of receptors to their ligands and their interaction with downstream signaling proteins. By suppressing PLC activities (Weber, C. *et al.* 1995), blocking PLA_2 effects (Grynberg, A. *et al.* 1992) and reducing the putative calcium-releasing compound inositol 1,4,5-trisphosphate (IP_3) (de Jonge, H.W. *et al.* 1996; Nair, S.S. *et al.* 2000), (n-3) PUFA reduce generation of arrhythmia and improve myocardial function recovery after ischemia-reperfusion. The (n-3) PUFA may also elicit antiarrhythmic effects by affecting major protein kinases such as protein kinase C (Bordoni, A. *et al.* 1992; Castillo, A. *et al.* 2005; Nyby, M.D. *et al.* 2003; Takahashi, R. *et al.* 2005), protein kinase A, calmodulin kinase II (Zaloga, G.P. *et al.* 2006) which can play an important roles in arrhythmia susceptibility (Anderson, M.E. *et al.* 1998; Zhang, R. *et al.* 2005) and sudden death (Pogwizd, S.M. & Bers, D.M. 2004). Furthermore, (n-3) PUFA affect kinase-mediated serine/threonine and tyrosine phosphorylation of cellular proteins (Chen, W. *et al.* 2007), a small G-protein, Rho, and its interaction with Rho kinase (Adamson, P. *et al.* 1992), and thereby eliciting electrical stability.

The prevention of mitochondrial calcium overload may also be an important mechanism of cardioprotection in ischemic preconditioning. Considerable evidence obtained from isolated rat heart studies has indicated an adaptation in calcium

regulation in preconditioned hearts. This is characterized by lower intracellular and mitochondrial calcium concentration with repeated brief ischemia episodes (Smith, G.B. *et al.* 1996), and reduced calcium concentrations during sustained longer periods of ischemia in preconditioned hearts (Murphy, E. *et al.* 1995; Steenbergen, C. *et al.* 1993; Tosaki, A. *et al.* 1994; Wang, Y.G. *et al.* 2001). Evidence suggests that preconditioning reduces an inappropriate release of calcium from the sarcoplasmic reticulum during sustained ischemia-reperfusion in the isolated rat heart (Zucchi, R. *et al.* 1995 Mubagwa, K. *et al.* 1997 Tani, M. *et al.* 1996). In addition, studies indicate that ischemic preconditioning may prevent cell death by inhibiting calcium influx secondary to intracellular acidosis. The resultant change in Na^+-H^+ exchange and influx of Na^+, leads to a subsequent increase in Na^+-Ca^{2+} exchange and increase in intracellular calcium (Asimakis, G.K. *et al.* 1992; Murphy, E. *et al.* 1995; Steenbergen, C. *et al.* 1993; Tosaki, A. *et al.* 1994). Furthermore, ischemic preconditioning blocks the postischemic burst of IP_3 production in isolated heart thereby preventing calcium overload and subsequent arrhythmia generation (Anderson, K.E. & Woodcock, E.A. 1995). These same authors observed that (n-3) PUFA also reduced ischemia-induced IP_3 production and cardiac arrhythmias (Anderson, K.E. *et al.* 1996), again providing a link between proposed mechanisms of ischemic-preconditioning and the (n-3) PUFA.

Many of the cardiac effects observed here have been attributed to subcellular events involving calcium. In turn, many subcellular calcium handling process have been shown to be influenced by (n-3) PUFA or by ischemic preconditioning or both. It will require further studies of these processes in dietary manipulated cells or whole heart to confirm their involvement individually or collectively.

Acute coronary occlusion, usually through atherosclerosis and thrombosis is the leading cause of death worldwide (Murray, C.J. & Lopez, A.D. 1997; WHO Feb,2007; Yusuf, S. *et al.* 2004), and it often occurs without prior recognition of the presence of cardiac disease (Fox, C.S. *et al.* 2004; Myerburg *et al.* 1993). In the early stages of an ischemic episode, patients are at high risk of sudden arrhythmic death. If a patient survives these early stages of a heart attack but ischemia is sustained for a sufficient period of time, myocardial infarction must inevitably occur. The morbidity and mortality of patients with myocardial infarction is determined by the severity of damage to the heart. Ischemic preconditioning represents a powerful experimental approach to reduce the severity of infarction by stimulating a range of endogenous mechanisms for cardioprotection (Murry, C.E. *et al.* 1986). Research focussing on identification of intracellular mediators and development of pharmacological mimetic agents of preconditioning to provide a therapeutic approach that might reliably protect the heart from ischemic insult (Downey, J.M. *et al.* 2007) has produced numerous examples that include adenosine, adenosine agonists, protein kinase C agonists, K_{ATP} channel openers, and NO donors (Downey, J.M. & Cohen, M.V. 1997). These agents are effective when given just prior to an ischemic episode to produce a genuine preconditioning effect, however, the need for administration of such agents immediately ahead of the onset of ischemia or reperfusion is a problem for clinical application, since the occurrence of an acute, out of hospital, ischemia event is rarely predictable. This limitation to ischemic preconditioning is recognized in the recent attention to the possibility of ischemic post-conditioning cardioprotection (use of agents after the ischemic event) (Baxter, G.F. & Burley, D.S. 2008). Furthermore, the potential

preconditioning agents are largely non-specific for myocardium and may produce unwanted side effects (Kloner, R.A. 2006; Kloner, R.A. & Rezkalla, S.H. 2006) unless administered by direct cardiac infusion.

In contrast to new pharmacological agents dietary fish oil has long been classified by the US-FDA as "generally regarded as safe" (GRAS) and has a long history of safe consumption associated with both primary and secondary cardioprotection. Human intervention trials demonstrated reduced mortality with regular intake of fish or fish oil, without changes in blood pressure, blood lipids and most significantly, despite not reducing the incidence of new cardiac events in normally high risk, post myocardial infarction patients (Kris-Etherton, P.M. *et al.* 2002).

Regardless of the convincing evidence of cardioprotective effects of fish oil, it was reported that western countries have decreased their overall fish intake over time, and are currently consuming less than the recommended amounts (Harper, C.R. & Jacobson, T.A. 2001). It has been estimated that the ratio of (n-6) PUFA:(n-3) PUFA in the diet of early humans was 1:1, today in the US this ratio has risen to ~10:1 (Simopoulos & P., A. 1999). The US and UK are currently consuming 0.1-0.2g/d of EPA and DHA which is well below the recommended intake of 0.65 and 1.5g/d in the US and UK, respectively (Harper, C.R. & Jacobson, T.A. 2001). The recommendations by the American Heart Association's Dietary Guidelines include at least two servings of fish, particularly fatty fish, per week. Patients with coronary heart disease should be encouraged to consume ~1g/d of EPA and DHA (Lichtenstein, A.H. *et al.* 2006). The fish oil consumed by rats in this study was

extracted from tuna fish without enrichment and although its high content of DHA relative to EPA (USFDA 2002) contrasts with the EPA-rich oils often used experimentally or for human supplementation (Bays, H. 2006), it does in fact reflect the balance of (n-3) PUFA found in many common table fish, including most salmon species, canned or fresh (Psota, T.L. *et al.* 2006; USDA 2007), in which DHA usually exceeds the EPA content. Furthermore, DHA is the predominant (n-3) PUFA obtained from fish in the usual human diet (Amiano, P. *et al.* 2001; Kris-Etherton, P.M. *et al.* 2000), and DHA is the principle (n-3) PUFA accumulated in hearts, even when purified EPA is fed (McLennan, P.L. *et al.* 1996) Therefore, the high-DHA tuna fish oil used in this study may better reflect the common food sources contributing to preventative cardioprotection in humans described in epidemiological studies and it directly provides the preferred cardiac (n-3) PUFA. It also reflects the numerous observation that DHA is often effective where EPA is not (McLennan, P.L. *et al.* 1996)

The results of the present thesis further emphasized the beneficial effects of (n-3) PUFA and it is hoped that this could raise more awareness of the importance of fish consumption as a preventive agent for healthy humans and therapeutic agent for treating cardiovascular abnormalities in some heart patients. In summary, the present thesis suggest that dietary fish oil may provide nutritional preconditioning, which would require no prediction or prior warning of ischemic events and thus represent a low-risk solution to ischemic cardioprotection. It may also help to explain the widely reported cardio protective effects already associated with regular fish or fish oil consumption.

Implications for Future Research

To obtain a more precise picture of how fish oil consumption and ischemic preconditioning exert their cardioprotective effects, further investigations are still required. The (n-3) PUFA seem to have multiple potential sites for modulating signal transduction pathways to alter cardiac functions. These pathways deserve further investigation. The endogenous antioxidant MnSOD may be involved in the cardioprotection afforded by both (n-3) PUFA and ischemic preconditioning. The increase in antioxidants may have inhibited the rise in oxidative products in ischemic preconditioned hearts and (n-3) PUFA treated hearts. However, since the incorporation of (n-3) PUFA and the increase in manganese superoxide dismutase is gradual, a dose response study of oxidation and antioxidant levels with higher or lower level of (n-3) PUFA should be investigated. Measurement of oxidation products and antioxidants at various time points after ischemic preconditioning stimuli and during early stages of reperfusion would more closely examine the potential cause and effect of oxidation, antioxidant activation and prevention of oxidation. In particular, the lag-time of membrane lipid oxidation (Dotan, Y. *et al.* 2004), which is a measure of resistance to peroxidation, may be altered. This can not be determined in the current study with only a single time point for evaluation of oxidation markers. It is also recommended that other biomarkers of oxidative damage be measured in addition to those already provided in this study. This would include measures of oxidative protein and DNA damage and other lipid peroxidation products such as conjugated dienes, alkanes, and 4-hydroxyyalkenals in addition to other antioxidants such as glutathione, and catalase. Measurements of 4-hydroxynoneal and 4-hydroxyhexanal, products of (n-6) PUFA and (n-3) PUFA

breakdown respectively are of particular importance in relation to fish oil consumption because they may help to determine if the EPA and DHA incorporated into cell membranes are themselves more readily oxidised (van Kuijk, F.J. *et al.* 1995). Furthermore, although low heart rate and low coronary flow with no deficit in cardiac function provide indirect evidence for the reduced oxygen consumption and increased efficiency of oxygen use that has previously been described from direct measures (Pepe, S. & McLennan, P.L. 2002, 2007), it would be valuable to measure myocardial oxygen consumption directly in future studies to establish whether oxygen use by the heart determines the contractile state of the myocardium.

In this study, the fish oil diet was designed to match the level of the total PUFA in (n-6) diet. This resulted in a high intake of (n-3) PUFA well above what can be achieved in human diet. Therefore, this work needs to be repeated to establish the effects of lower (n-3) PUFA intake. However, earlier studies that observed cardioprotective effects of (n-3) PUFA in isolated heart (Pepe, S. & McLennan, P.L. 2002) used higher intake of fish oil (12%) compared to 7% used in this study. When this work was repeated at low level of (n-3) PUFA, the cardioprotective effects of (n-3) PUFA were still evident (Pepe, S. & McLennan, P.L, 2007). Similarly, the antiarrhythmic effect first observed with high intakes of fish oil (McLennan, P.L. *et al.* 1988) have been reported at lower intakes (McLennan, P.L. *et al.* 2007). Therefore, there is good reason to believe the effects observed in the present study can be obtained with lower fish oil intakes in the future.

In addition to further mechanistic studies of preconditioning effect of (n-3) PUFA, the possible postconditioning effect of (n-3) PUFA also deserves investigation. Ischemic postconditioning is defined as application of one or more episodes of short-lived reperfusion and ischaemic episodes at the end of the index ischaemia period at the immediate onset of myocardial reperfusion. Ischemic postconditioning has already been demonstrated to be an effective cardioprotective intervention in patients undergoing primary percutaneous coronary intervention for their acute myocardial infarction. However, because of the invasive nature of the ischemic postconditioning protocol and the restriction of its use to patients undergoing primary percutaneous coronary intervention, new strategies for cardioprotection are still required. The demonstration in rats that the n-3 PUFA cardioprotection evidenced in this study could be additive to postconditioning, or as efficient as postconditioning would be a striking clinical issue since a majority of the patients submitted to primary percutaneous coronary intervention may not be eligible for postconditioning due to their clinical conditions.

Summary of Primary Findings

A comprehensive list of the findings of this thesis is given as follows:

- Six weeks treatment of rats with an (n-3) PUFA enriched diet caused substantial alterations in myocardial phospholipid fatty acid composition (Chapter Four).

- DHA was largely incorporated into heart phospholipid membrane when the diet had been supplemented with fish oil (Chapter Four).

- This increased incorporation of DHA into the heart membrane was associated with decreased concentrations of linoleic and arachidonic acid (Chapter Four).

- Myocardial membranes of fish oil hearts exhibited an increased in peroxidization index, which suggested an increase in susceptibility of the membrane to oxidative damage (Chapter Four).

- Acute ischemic preconditioning did not modify the membrane fatty acid composition under any administered diets (Chapter Four).

- Feeding (n-3) PUFA significantly reduced myocardial infarct size after ischemia-reperfusion in isolated hearts. (Chapter Five).

- Ischemic preconditioning significantly reduced infarct size after ischemia-reperfusion in SF hearts and (n-6) PUFA hearts but did not further reduce infarct size in (n-3) PUFA hearts (Chapter Five).

- Both (n-3) PUFA diet and ischemic-preconditioning reduced coronary flow at rest and during reperfusion, indicative of reduced myocardial oxygen delivery without detriment to cardiac function (Chapter Five).

- The (n-3) PUFA caused reduction in heart rate, left ventricular end diastolic pressure and an enhanced left ventricular developed pressure (Chapter Five).

- Ischemic preconditioning also caused reductions in heart rate, left ventricular end diastolic pressure and an enhanced left ventricular developed pressure (Chapter Five).

- Fish oil hearts reduced arrhythmia in both ischemia and reperfusion.

- Ischemic preconditioning also reduced arrhythmia in both ischemia and reperfusion. Although ischemic preconditioning reduced arrhythmia under all conditions, ischemic preconditioned hearts were significantly less arrhythmic if they also had (n-3) PUFA incorporation.

- Fish oil hearts had higher basal concentration of the markers of lipid oxidation, lipid hydroperoxide and malondialdehyde (Chapter Six).

- Ischemic stress-induced increase in lipid oxidation was inhibited by both (n-3) PUFA and ischemic preconditioning (Chapter Six).

- Fish oil induced higher concentrations of superoxide dismutase in normoxic heart tissues specifically due to increased mitochondrial manganese superoxide dismutase (Chapter Six).

- Ischemic preconditioning also induced an increase in manganese superoxide dismutase in SF and (n-6) PUFA hearts but did not further increase concentration in (n-3) PUFA hearts (Chapter Six).

- While both (n-3) PUFA and ischemic preconditioning protected the heart against the adverse effects of prolonged ischemia, the effects were not additive or synergistic (Chapter Five& Chapter Six).

- Fish oil appears to produce "dietary" or "nutritional" preconditioning equivalent to ischemic preconditioning in terms of cardioprotection (Chapter Five& Chapter Six).

LITERATURE CITED

Adamson, P., Marshall, C.J., Hall, A., and Tilbrook, P.A. (1992) Post-translational modifications of p21rho proteins. *J. Biol. Chem.* **267**: 20033-38.

Agren, J.J., Vaisanen, S., Hanninen, O., Muller, A.D., and Hornstra, G. (1997) Hemostatic factors and platelet aggregation after a fish-enriched diet or fish oil or docosahexaenoic acid supplementation. *Prostaglandins Leukot. Essent. Fatty Acids.* **57**: 419-21.

Al Makdessi, S., Sweidan, H., and Jacob, R. (1994) (n-3) versus (n-6) fatty acid incorporation into the phospholipids of rat heart sarcolemma. A comparative study of four different oil diets. *J. Mol. Cell. Cardiol.* **26**: 23-29.

Al Makdessi, S., Brandle, M., Ehrt, M., Sweidan, H., and Jacob, R. (1995) Myocardial protection by ischemic preconditioning: The influence of the composition of myocardial phospholipids. *Mol. Cell. Biochem.* **145**: 69-73.

Albert, C.M., Campos, H., Stampfer, M.J., Ridker, P.M., Manson, J.E., Willett, W.C., and Ma, J. (2002) Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *N. Engl. J. Med.* **346**: 1113-18.

Albert, C.M., Hennekens, C.H., O'Donnell, C.J., Ajani, U.A., Carey, V.J., Willett, W.C., Ruskin, J.N., et al. (1998) Fish consumption and risk of sudden cardiac death. *JAMA.* **279**: 23-28.

Alderman, M.H., Madhavan, S., and Cohen, H. (1996) Antihypertensive drug therapy the effect of jnc criteria on prescribing patterns and patient status through the first year. *Am. J. Hypertens.* **9**: 413-18.

Alphabetical listing of GRAS Notices. US. FDA Centre for Food safety and Applied Nutrition, Office of Food Additive safety; c2008 [cited 2009 September]. Available from: http://www.cfsan.fda.gov/~rdb/opagras1.html

Allibardi, S., Merati, G., Chierchia, S., and Samaja, M. (1999) Atenolol depresses post-ischemic recovery in the isolated rat heart. *Pharmacol. Res.* **39**: 431-35.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., et al. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* **271**: 20246-49.

Amiano, P., Dorronsoro, M., de Renobales, M., Ruiz de Gordoa, J.C., and Irigoien, I. (2001) Very-long-chain omega-3 fatty acids as markers for habitual fish intake in a population consuming mainly lean fish: The epic cohort of gipuzkoa. European prospective investigation into cancer and nutrition. *Eur. J. Clin. Nutr.* **55**: 827-32.

Anderson, K.E. and Woodcock, E.A. (1995) Preconditioning of perfused rat heart inhibits reperfusion-induced release of inositol (1,4,5) trisphosphate. *J. Mol. Cell. Cardiol.* **27**: 2421-31.

Anderson, K.E., Du, X.J., Sinclair, A.J., Woodcock, E.A., and Dart, A.M. (1996) Dietary fish oil prevents reperfusion ins (1,4,5) p_3 release in rat heart: Possible antiarrhythmic mechanism. *Am J Physiol H.* **271**: H1483-90.

Anderson, M.E., Braun, A.P., Wu, Y., Lu, T., Wu, Y., Schulman, H., and Sung, R.J. (1998) KN-93, an inhibitor of multifunctional Ca^{++}/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. *J. Pharmacol. Exp. Ther.* **287**: 996-1006.

Anderson, R.E., Maude, M.B., and Feldman, G.L. (1970) Lipids of ocular tissues. 3. The phospholipids of mature bovine iris. *Exp. Eye Res.* **9**: 281-4.

Ando, K., Nagata, K., Yoshida, R., Kikugawa, K., and Suzuki, M. (2000) Effect of n−3 polyunsaturated fatty acid supplementation on lipid peroxidation of rat organs. *Lipids.* **35**: 401-07.

Andrade, S.E., Walker, A.M., Gottlieb, L.K., Hollenberg, N.K., Testa, M.A., Saperia, G.M., and Platt, R. (1995) Discontinuation of antihyperlipidemic drugs -- do rates reported in clinical trials reflect rates in primary care settings? *N. Engl. J. Med.* **332**: 1125-31.

Appel, L.J., Miller, E.R., 3rd, Seidler, A.J., and Whelton, P.K. (1993) Does supplementation of diet with 'fish oil' reduce blood pressure? A meta-analysis of controlled clinical trials. *Arch. Intern. Med.* **153**: 1429-38.

Argaud, L., Gateau-Roesch, O., Chalabreysse, L., Gomez, L., Loufouat, J., Thivolet-Bejui, F., Robert, D., et al. (2004) Preconditioning delays Ca^{2+}-induced mitochondrial permeability transition. *Cardiovasc Res.* **61**: 115-22.

Armstrong, S.C., Delacey, M., and Ganote, C.E. (1999) Phosphorylation state of hsp27 and p38 mapk during preconditioning and protein phosphatase inhibitor protection of rabbit cardiomyocytes. *J. Mol. Cell. Cardiol.* **31**: 555-67.

Armstrong, S.C., Shivell, L.C., and Ganote, C.E. (2001) Sarcolemmal blebs and osmotic fragility as correlates of irreversible ischemic injury in preconditioned isolated rabbit cardiomyocytes. *J. Mol. Cell. Cardiol.* **33**: 149-60.

Asemu, G., Dhalla, N.S., and Tappia, P.S. (2004) Inhibition of PLC improves postischemic recovery in isolated rat heart. *Am J Physiol Heart Circ Physiol.* **287**: H2598-605.

Asimakis, G.K., Inners-McBride, K., Medellin, G., and Conti, V.R. (1992) Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *Am J Physiol Heart Circ Physiol.* **263**: H887-94.

Auchampach, J.A., Rizvi, A., Qiu, Y., Tang, X.-L., Maldonado, C., Teschner, S., and Bolli, R. (1997) Selective activation of A_3 adenosine receptors with n6-(3-iodobenzyl) adenosine-5'-n-methyluronamide protects against myocardial stunning and infarction without hemodynamic changes in conscious rabbits. *Circ. Res.* **80**: 800-09.

Awad, A.B. and Chattopadhyay, J.P. (1983) Effect of dietary fats on the lipid composition and enzyme activities of rat cardiac sarcolemma. *J. Nutr.* **113**: 1878-83.

Ayalew-Pervanchon, A., Rousseau, D., Moreau, D., Assayag, P., Weill, P., and Grynberg, A. (2007) Long-term effect of dietary {alpha}-linolenic acid or decosahexaenoic acid on incorporation of decosahexaenoic acid in membranes and its influence on rat heart in vivo. *Am J Physiol Heart Circ Physiol.* **293**: H2296-304.

Backer, G.D., Ambrosini, E., Borch-Johnsen, K., Brotons, C., Cifkova, R., and Dallongeville, J. (2003) European guidelines on cardiovascular disease prevention in clinical practice. Third joint task force of european and other societies on cardiovascular disease prevention in clinical practice. *Eur Heart.* **24**: 1601-10.

Bagchi, M., Prasad, M.R., Engelman, R.M., and Das, D.K. (1989) Effects of free radicals on the fluidity of myocardial membranes. *Free Radic. Res. Commun.* **7**: 375-80.

Baines, C.P., Goto, M., and Downey, J.M. (1997) Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J. Mol. Cell. Cardiol.* **29**: 207-16.

Baker, H.J., Lindsey, J.R., and Weisbroth, S.H. (1979) The laboratory rat. *Biology and Diseases.* **Volume 1** (New York, Academic Press): 123-152

Banerjee, A., Locke-Winter, C., Rogers, K.B., Mitchell, M.B., Brew, E.C., Cairns, C.B., Bensard, D.D., et al. (1993) Preconditioning against myocardial dysfunction after ischemia and reperfusion by an alpha 1-adrenergic mechanism. *Circ. Res.* **73**: 656-70.

Bang, H.O., Dyerberg, J., and Hjoorne, N. (1976) The composition of food consumed by greenland eskimos. *Acta Med. Scand.* **200**: 69-73.

Barbosa, D.S., Cecchini, R., El Kadri, M.Z., Rodriguez, M.A., Burini, R.C., and Dichi, I. (2003) Decreased oxidative stress in patients with ulcerative colitis supplemented with fish oil omega-3 fatty acids. *Nutrition.* **19**: 837-42.

Barcelli, U., Glas-Greenwalt, P., and Pollak, V.E. (1985) Enhancing effect of dietary supplementation with omega-3 fatty acids on plasma fibrinolysis in normal subjects. *Thromb. Res.* **39**: 307-12.

Bartling, B., Holtz, J., and Darmer, D. (1998) Contribution of myocyte apoptosis to myocardial infarction? *Basic Res. Cardiol.* **93**: 71-84.

Bashore, T.M., Granger, C.B., and Patel, M.R. (2009), *Current medical diagnosis and treatment*, Heart Disease**,** eds McPhee, S.J., Papadakis, M.A., and Tierney, L.M. (48e edn.; USA: Mcgraw-Hill). http://www.accessmedicine.com/content.aspx?aID=3671.

Baur, L.A., O'Connor, J., Pan, D.A., Wu, B.J., O'Connor, M.J., and Storlien, L.H. (2000) Relationships between the fatty acid composition of muscle and erythrocyte membrane phospholipid in young children and the effect of type of infant feeding. *Lipids.* **35**: 77-82.

Baxter, G.F. (2002) Role of adenosine in delayed preconditioning of myocardium. *Cardiovasc Res.* **55**: 483-94.

Baxter, G.F. and Yellon, D.M. (1999) ATP-sensitive K^+ channels mediate the delayed cardioprotective effect of adenosine a1 receptor activation. *J. Mol. Cell. Cardiol.* **31**: 981-9.

Baxter, G.F. and Ferdinandy, P. (2001) Delayed preconditioning of myocardium: Current perspectives. *Basic Res. Cardiol.* **96**: 329-44.

Baxter, G.F. and Ebrahim, Z. (2002) Role of bradykinin in preconditioning and protection of the ischaemic myocardium. *Br. J. Pharmacol.* **135**: 843-54.

Baxter, G.F. and Burley, D.S. (2008) Reperfusion and calculated risks: Pharmacological postconditioning of human myocardium. *Br. J. Pharmacol.* **153**: 1-3.

Baxter, G.F., Goma, F.M., and Yellon, D.M. (1997) Characterisation of the infarct-limiting effect of delayed preconditioning: Timecourse and dose-dependency studies in rabbit myocardium. *Basic Res. Cardiol.* **92**: 159-67.

Baxter, G.F., Marber, M.S., Patel, V.C., and Yellon, D.M. (1994) Adenosine receptor involvement in a delayed phase of myocardial protection 24 hours after ischemic preconditioning. *Circulation.* **90**: 2993-3000.

Bays, H. (2006) Clinical overview of omacor: A concentrated formulation of omega-3 polyunsaturated fatty acids. *Am. J. Cardiol.* **98**: 71i-76i.

Beckman, K.B. and Ames, B.N. (1998) The free radical theory of aging matures. *Physiol. Rev.* **78**: 547-81.

Belhomme, D., Peynet, J., Florens, E., Tibourtine, O., Kitakaze, M., and Menasche, P. (2000) Is adenosine preconditioning truly cardioprotective in coronary artery bypass surgery? *Ann. Thorac. Surg.* **70**: 590-94.

Bell, R.M., Smith, C.C., and Yellon, D.M. (2002) Nitric oxide as a mediator of delayed pharmacological a(1) receptor triggered preconditioning; is eNOS masquerading as iNOS? *Cardiovasc Res.* **53**: 405-13.

Benzi, G. (1993) Aerobic performance and oxygen free-radicals. *J. Sports Med. Phys. Fitness.* **33**: 205-22.

Bernardo, N.L., D'Angelo, M., Okubo, S., Joy, A., and Kukreja, R.C. (1999) Delayed ischemic preconditioning with adenosine is mediated by opening

of ATP-sensitive K^+ channels in the rabbit heart. *Am J Physiol Heart Circ Physiol.* **276**: H1323-30.

Berridge, M.J. (1997) Elementary and global aspects of calcium signaling. *J. Physiol.* **499**: 291-306.

Bhatnagar, A., Srivastava, S.K., and Szabo, G. (1990) Oxidative stress alters specific membrane currents in isolated cardiac myocytes. *Circ. Res.* **67**: 535-49.

Bian, J.S., Zhang, W.M., Xia, Q., and Wong, T.M. (1998) Phospholipase c inhibitors attenuate arrhythmias induced by kappa-receptor stimulation in the isolated rat heart. *J. Mol. Cell. Cardiol.* **30**: 2103-10.

Billman, G.E., Kang, J.X., and Leaf, A. (1999) Prevention of sudden cardiac death by dietary pure {omega}-3 polyunsaturated fatty acids in dogs. *Circulation.* **99**: 2452-57.

Bjerregaard, P., Mulvad, G., and Pedersen, H.S. (1997) Cardiovascular risk factors in inuit of greenland. *Int. J. Epidemiol.* **26**: 1182-90.

Blumgart, H.L., Gilligan, D.R., and Schlesinger, M.J. (1941) Experimental studies on the effect of temporary occlusion of coronary arteries and the production of myocardial infarction. *Am. Heart J.* **22**: 374-89.

Bolli, R. (1991) Mechanisms of myocardial "Stunning". *J. Cardiothorac. Vasc. Anesth.* **5**: 188-88.

Bolli, R. (1992) Myocardial 'stunning' in man. *Circulation.* **86**: 1671-91.

Bolli, R. (2000) The late phase of preconditioning. *Circ. Res.* **87**: 972-83.

Bolli, R. and Marban, E. (1999) Molecular and cellular mechanisms of myocardial stunning. *Physiol. Rev.* **79**: 609-34.

Bolli, R., Jeroudi, M.O., Patel, B.S., DuBose, C.M., Lai, E.K., Roberts, R., and McCay, P.B. (1989) Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *PNAS.* **86**: 4695-99.

Bolli, R., Shinmura, K., Tang, X.L., Kodani, E., Xuan, Y.T., Guo, Y., and Dawn, B. (2002) Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovasc Res.* **55**: 506-19.

Bolli, R., Dawn, B., Tang, X.L., Qiu, Y., Ping, P., Xuan, Y.T., Jones, W.K., et al. (1998) The nitric oxide hypothesis of late preconditioning. *Basic Res. Cardiol.* **93**: 325-38.

Bonaa, K.H., Bjerve, K.S., and Nordoy, A. (1992a) Docosahexaenoic and eicosapentaenoic acids in plasma phospholipids are divergently associated with high density lipoprotein in humans. *Arterioscler. Thromb.* **12**: 675-81.

Bonaa, K.H., Bjerve, K.S., and Nordoy, A. (1992b) Habitual fish consumption, plasma phospholipid fatty acids, and serum lipids: The tromso study. *Am. J. Clin. Nutr.* **55**: 1126-34.

Bordoni, A., Biagi, P.L., Turchetto, E., Rossi, C.A., and Hrelia, S. (1992) Diacylglycerol fatty acid composition is related to activation of protein kinase c in cultured cardiomyocytes. *Cardioscience.* **3**: 251-5.

Botham, K. and Mayes, P. (2009A), 'Harper's illustrated biochemistry', in Robert K. Murray, D.K.G., Victor W. Rodwell (ed.), *Biosynthesis of Fatty Acids & Eicosanoids* (28e edn.; Columbus: The McGraw-Hill Companies). http://www.accessmedicine.com/content.aspx?aID=5227280.

Botham, K. and Mayes, P. (2009B), 'Harper's illustrated biochemistry', in Robert K. Murray, D.K.G., Victor W. Rodwell (ed.), *Oxidation of fatty acids: Ketogenesis* (28e edn.; Columbus: The McGraw-Hill Companies). http://www.accessmedicine.com/content.aspx?aID=5227210.

Botham, K. and Mayes, P. (2009C), 'Harper's illustrated biochemistry', in Robert K. Murray, D.K.G., Victor W. Rodwell (ed.), *Biologic Oxidation* (28e edn.; Columbus: The McGraw-Hill Companies). http://www.accessmedicine.com/content.aspx?aID=5226463.

Bourre, J.M., Piciotti, M., Dumont, O., Pascal, G., and Durand, G. (1990) Dietary linoleic acid and polyunsaturated fatty acids in rat brain and other organs. Minimal requirements of linoleic acid. *Lipids.* **25**: 465-72.

Brandes, R. and Bers, D.M. (1997) Intracellular Ca^{2+} increases the mitochondrial NADH concentration during elevated work in intact cardiac muscle. *Circ. Res.* **80**: 82-87.

Braunwald, E. (1993) The open-artery theory is alive and well -- again. *N. Engl. J. Med.* **329**: 1650-52.

Braunwald, E. and Kloner, R.A. (1982) The stunned myocardium: Prolonged, postischemic ventricular dysfunction. *Circulation.* **66**: 1146-49.

Braunwald, E. and Kloner, R.A. (1985) Myocardial reperfusion: A double-edged sword? *J. Clin. Invest.* **76**: 1713-9.

Braunwald, E., Sarnoff, S.J., Case, R.B., Stainsby, W.N., and Welch, G.H.J. (1957) Hemodynamic determinants of coronary flow: Effect of changes in aortic pressure and cardiac output on the relationship between myocardial oxygen consumption and coronary flow. *Am. J. Physiol.* **192**: 157-63.

Brenner, R.R. (1971) The desaturation step in the animal biosynthesis of polyunsaturated fatty acids. *Lipids.* **6**: 567-75.

Brenner, R.R. (1981) Nutritional and hormonal factors influencing desaturation of essential fatty acids. *Prog. Lipid Res.* **20**: 41-7.

Brooks, G. and Hearse, D.J. (1996) Role of protein kinase c in ischemic preconditioning: Player or spectator? *Circ. Res.* **79**: 628-31.

Brooks, W.W., Conrad, C.H., and Morgan, J.P. (1995) Reperfusion induced arrhythmias following ischaemia in intact rat heart: Role of intracellular calcium. *Cardiovasc Res.* **29**: 536-42.

Brouwer, M.A., Martin, J.S., Maynard, C., Wirkus, M., Litwin, P.E., Verheugt, F.W., and Weaver, W.D. (1996) Influence of early prehospital thrombolysis on mortality and event-free survival (the myocardial infarction triage and intervention [MITI] randomized trial). MITI project investigators. *Am. J. Cardiol.* **78**: 497-502.

Buckley, J.D., Burgess, S., Murphy, K.J., and Howe, P.R. (2008) DHA-rich fish oil lowers heart rate during submaximal exercise in elite australian rules footballers. *J. Sci. Med. Sport.* **12**: 503-507.

Budowski, P. and Crawford, M.A. (1985) Alpha-linolenic acid as a regulator of the metabolism of arachidonic acid: Dietary implications of the ratio, n-6:n-3 fatty acids. *Proc. Nutr. Soc.* **44**: 221-9.

Burdge, G.C., Wright, P., Jones, A.E., and Wootton, S.A. (2000) A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. *Br. J. Nutr.* **84**: 781-7.

Burke, A. and Renu, V. (2008), 'Hurst's the heart ', in Walsh, R.A. and Simon, D.I. (12e eds.), *Pathology of myocardial ischemia, infarction, reperfusion, and sudden death* (Oklahoma: McGraw- Hill). http://www.accessmedicine.com/content.aspx?aID=3054555.

Burns, P.G., Krukenkamp, I.B., Caldarone, C.A., Gaudette, G.R., Bukhari, E.A., and Levitsky, S. (1995) Does cardiopulmonary bypass alone elicit myoprotective preconditioning? *Circulation.* **92**: 447-51.

Burr, M.L., Fehily, A.M., and Gilbert, J.F. (1989a) Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and reinfarction trial (DART). *Lancet.* **2**: 757-61.

Burr, M.L., Fehily, M., Rogers, S., Welsby, E., King, S., and Sandham, S. (1989b) Diet and reinfarction trial (DART): Design, recruitment, and compliance. *Eur. Heart J.* **10**: 558-67.

Calder, P.C. (2006) n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr.* **83**: S1505-19.

Cannon, C.P. (1999) Optimizing the medical management of acute coronary syndromes. *J. Thromb. Thrombolysis.* **7**: 171-89.

Carafoli, E. (1987) Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56**: 395-433.

Cardiovascular disease fact sheet. World Health Organization;c2007 [cited 2009 February]. Available from http://apps.who.int/mediacentre/factsheets/fs317/en/index.html

Carini, R., Parola, M., Dianzani, M.U., and Albano, E. (1992) Lipid peroxidation and hepatocyte death investigation of a possible mechanism of oxidative cell injury. *Ann. N. Y. Acad. Sci.* **663**: 444-6.

Carmeliet, E. (1999) Cardiac ionic currents and acute ischemia: From channels to arrhythmias. *Physiol. Rev.* **79**: 917-1017.

Carrozza, J.P., Jr., Bentivegna, L.A., Williams, C.P., Kuntz, R.E., Grossman, W., and Morgan, J.P. (1992) Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. *Circ. Res.* **71**: 1334-40.

Castillo, A., Ruzmetov, N., Harvey, K., Stillwell, W., Zaloga, G., and Siddiqui, R. (2005) Docosahexaenoic acid inhibits protein kinase c translocation/activation and cardiac hypertrophy in rat cardiomyocytes. *J Mol Genet Med.* **1**: 18-25.

Cave, A.C. and Hearse, D.J. (1992) Ischaemic preconditioning and contractile function: Studies with normothermic and hypothermic global ischaemia. *J. Mol. Cell. Cardiol.* **24**: 1113-23.

Chandrasekar, B. and Fernandes, G. (1994) Decreased proinflammatory cytokines and increased antioxidant enzyme gene expression by [omega]-3 lipids in murine lupus nephritis. *Biochem. Biophys. Res. Commun.* **200**: 893-98.

Charnock, J.S., Abeywardena, M.Y., and McLennan, P.L. (1986) Comparative changes in the fatty-acid composition of rat cardiac phospholipids after long-term feeding of sunflower seed oil- or tuna fish oil-supplemented diets. *Ann. Nutr. Metab.* **30**: 393-406.

Charnock, J.S., Abeywardena, M.W., and McLennan, P.L. (1989) Tissue specific differences in the fatty acid composition of the marmoset monkey (callithrix jacchus). *Comp Biochem Physiol A Comp Physiol.* **92**: 299-304.

Charnock, J.S., McLennan, P.L., Abeywardena, M.Y., and Russell, G.R. (1985) Altered levels of n-6/n-3 fatty acids in rat heart and storage fat following variable dietary intake of linoleic acid. *Ann. Nutr. Metab.* **29**: 279-88.

Charnock, J.S., Abeywardena, M.Y., Poletti, V.M., and McLennan, P.L. (1992) Differences in fatty acid composition of various tissues of the marmoset monkey (callithrix jacchus) after different lipid supplemented diets. *Comp. Biochem. Physiol. Comp. Physiol.* **101**: 387-93.

Charnock, J.S., Dryden, W.F., McMurchie, E.J., Abeywardena, M.Y., and Russell, G.R. (1983) Differences in the fatty acid composition of atrial and ventricular phospholipids of rat heart following standard and lipid-supplemented diets. *Comp. Biochem. Physiol. B.* **75**: 47-52.

Chautan, M., Calaf, R., Leonardi, J., Charbonnier, M., Andre, M., Portugal, H., Pauli, A.M., et al. (1990) Inverse modifications of heart and liver alpha-tocopherol status by various dietary n-6/n-3 polyunsaturated fatty acid ratios. *J. Lipid Res.* **31**: 2201-8.

Chen, W., Gabel, S., Steenbergen, C., and Murphy, E. (1995) A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ. Res.* **77**: 424-9.

Chen, W., Jump, D.B., Esselman, W.J., and Busik, J.V. (2007) Inhibition of cytokine signaling in human retinal endothelial cells through modification of caveolae/lipid rafts by docosahexaenoic acid. *Invest. Ophthalmol. Vis. Sci.* **48**: 18-26.

Chen, Z., Siu, B., Ho, Y.S., Vincent, R., Chua, C.C., Hamdy, R.C., and Chua, B.H. (1998) Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J. Mol. Cell. Cardiol.* **30**: 2281-9.

Cho, S.Y., Miyashita, K., Miyazawa, T., Fujimoto, K., and Kanada, T. (1978) Autooxidation of ethyl eicosapentaenoate and docosahexaenoate. *J. Am. Oil Chem. Soc.* **64**: 876-79.

Cleveland, J.C., Meldrum, D.R., Rowland, R.T., Banerjee, A., and Harken, A.H. (1997) Adenosine preconditioning of human myocardium is dependent upon the ATP-sensitive K^+ channel. *J. Mol. Cell. Cardiol.* **29**: 175-82.

Cleveland, J.C., Jr., Wollmering, M.M., Meldrum, D.R., Rowland, R.T., Rehring, T.F., Sheridan, B.C., Harken, A.H., et al. (1996) Ischemic preconditioning in human and rat ventricle. *Am J Physiol Heart Circ Physiol.* **271**: H1786-94.

Cohen, J.S. (2001) Adverse drug effects, compliance, and initial doses of antihypertensive drugs recommended by the joint National committee vs the physicians' desk Reference. *Arch. Intern. Med.* **161**: 880-85.

Cohen, M.V., Liu, G.S., and Downey, J.M. (1991) Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation.* **84**: 341-9.

Cohen, M.V., Yang, X.M., and Downey, J.M. (1994) Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning. *Circ. Res.* **74**: 998-1004.

Cohen, M.V., Baines, C.P., and Downey, J.M. (2000) Ischemic preconditioning: From adenosine receptor to KATP channel. *Annu. Rev. Physiol.* **62**: 79-109.

Cohen, M.V., Walsh, R.S., Goto, M., and Downey, J.M. (1995) Hypoxia preconditions rabbit myocardium via adenosine and catecholamine release. *J. Mol. Cell. Cardiol.* **27**: 1527-34.

Collen, D. (1996) Fibrin-selective thrombolytic therapy for acute myocardial infarction. *Circulation.* **93**: 857-65.

Conquer, J.A., Roelfsema, H., Zecevic, J., Graham, T.E., and Holub, B.J. (2002) Effect of exercise on fatty acid profiles in (n-3) fatty acid -supplemented and -nonsupplemented premenopausal women. *Lipids.* **37**: 947-51.

Cunnane, S.C. and Anderson, M.J. (1997) The majority of dietary linoleate in growing rats is beta -oxidized or stored in visceral fat. *J. Nutr.* **127**: 146-52.

Curtis, M.J. (1998) Characterisation, utilisation and clinical relevance of isolated perfused heart models of ischaemia-induced ventricular fibrillation. *Cardiovasc Res.* **39**: 194-215.

Curtis, M.J., Macleod, B.A., Tabrizchi, R., and Walker, M.J. (1986) An improved perfusion apparatus for small animal hearts. *J. Pharmacol. Methods.* **15**: 87-94.

da Silva, M.M., Sartori, A., Belisle, E., and Kowaltowski, A.J. (2003) Ischemic preconditioning inhibits mitochondrial respiration, increases H_2O_2 release, and enhances K^+ transport. *Am J Physiol Heart Circ Physiol.* **285**: H154-62.

Dallongeville, J., Yarnell, J., Ducimetiere, P., Arveiler, D., Ferrieres, J., Montaye, M., Luc, G., et al. (2003) Fish consumption is associated with lower heart rates. *Circulation.* **108**: 820-25.

Dana, A., Baxter, G.F., and Yellon, D.M. (2001) Delayed or second window preconditioning induced by adenosine a1 receptor activation is independent of early generation of nitric oxide or late induction of inducible nitric oxide synthase. *J. Cardiovasc. Pharmacol.* **38**: 278-87.

Dana, A., Baxter, G.F., Walker, J.M., and Yellon, D.M. (1998) Prolonging the delayed phase of myocardial protection: Repetitive adenosine a1 receptor activation maintains rabbit myocardium in a preconditioned state. *J. Am. Coll. Cardiol.* **31**: 1142-49.

Danthi, S.J., Enyeart, J.A., and Enyeart, J.J. (2005) Modulation of native T-type calcium channels by omega-3 fatty acids. *Biochem. Biophys. Res. Commun.* **327**: 485-93.

Das, D.K., Engelman, R.M., and Kimura, Y. (1993) Molecular adaptation of cellular defences following preconditioning of the heart by repeated ischaemia. *Cardiovasc Res.* **27**: 578-84.

Das, D.K., Maulik, N., and Moraru, II (1995) Gene expression in acute myocardial stress. Induction by hypoxia, ischemia, reperfusion, hyperthermia and oxidative stress. *J. Mol. Cell. Cardiol.* **27**: 181-93.

Das, D.K., Maulik, N., Sato, M., and Ray, P.S. (1999) Reactive oxygen species function as second messenger during ischemic preconditioning of heart. *Mol. Cell. Biochem.* **196**: 59-67.

Daviglus, M.L., Stamler, J., Orencia, A.J., Dyer, A.R., Liu, K., Greenland, P., Walsh, M.K., et al. (1997) Fish consumption and the 30-year risk of fatal myocardial infarction. *N. Engl. J. Med.* **336**: 1046-53.

Dawn, B. and Bolli, R. (2002) Role of nitric oxide in myocardial preconditioning. *Ann. N. Y. Acad. Sci.* **962**: 18-41.

de Gomez Dumm, I.N. and Brenner, R.R. (1975) Oxidative desaturation of alpha-linoleic, linoleic, and stearic acids by human liver microsomes. *Lipids.* **10**: 315-7.

De Jonge, H.W., Van Heugten, H.A., and Lamers, J.M. (1995) Signal transduction by the phosphatidylinositol cycle in myocardium. *J. Mol. Cell. Cardiol.* **27**: 93-106.

de Jonge, H.W., Dekkers, D.H., Bastiaanse, E.M., Bezstarosti, K., van der Laarse, A., and Lamers, J.M. (1996) Eicosapentaenoic acid incorporation in membrane phospholipids modulates receptor-mediated phospholipase c and membrane fluidity in rat ventricular myocytes in culture. *J. Mol. Cell. Cardiol.* **28**: 1097-108.

de Lorgeril, M., Salen, P., Martin, J.-L., Monjaud, I., Delaye, J., and Mamelle, N. (1999) Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: Final report of the Lyon Diet Heart study. *Circulation.* **99**: 779-85.

de Lorgeril, M., Renaud, S., Mamelle, N., Salen, P., Martin, J.L., Monjaud, I., Guidollet, J., et al. (1994) Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet.* **343**: 1454-9.

de Zwart, L.L., Meerman, J.H., Commandeur, J.N., and Vermeulen, N.P. (1999) Biomarkers of free radical damage applications in experimental animals and in humans. *Free Radic. Biol. Med.* **26**: 202-26.

Demaison, L., Blet, J., Sergiel, J.P., Gregoire, S., and Argaud, D. (2000) Effect of dietary polyunsaturated fatty acids on contractile function of hearts isolated from sedentary and trained rats. *Reprod. Nutr. Dev.* **40**: 113-25.

Demaison, L., Moreau, D., Vergely-Vandriesse, C., Gregoire, S., Degois, M., and Rochette, L. (2001) Effects of dietary polyunsaturated fatty acids and hepatic steatosis on the functioning of isolated working rat heart under normoxic conditions and during post-ischemic reperfusion. *Mol. Cell. Biochem.* **224**: 103-16.

Demoz, A., Asiedu, D.K., Lie, O., and Berge, R.K. (1994) Modulation of plasma and hepatic oxidative status and changes in plasma lipid profile by n-3 (EPA and DHA), n-6 (corn oil) and a 3-thia fatty acid in rats. *Biochim. Biophys. Acta.* **1199**: 238-44.

Den Ruijter, H.M., Berecki, G., Opthof, T., Verkerk, A.O., Zock, P.L., and Coronel, R. (2007) Pro- and antiarrhythmic properties of a diet rich in fish oil. *Cardiovasc Res.* **73**: 316-25.

Denton, R.M. and McCormack, J.G. (1990) Ca^{2+} as a second messenger within mitochondria of the heart and other tissues. *Annu. Rev. Physiol.* **52**: 451-66.

Deutsch, E., Berger, M., Kussmaul, W.G., Hirshfeld, J.W., Jr., Herrmann, H.C., and Laskey, W.K. (1990) Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic, and metabolic features. *Circulation.* **82**: 2044-51.

Dewailly, E., Blanchet, C., Gingras, S., Lemieux, S., and Holub, B.J. (2002) Cardiovascular disease risk factors and n-3 fatty acid status in the adult population of James Bay Cree. *Am. J. Clinical Nutrition.* **76**: 85-92.

DeWood, M.A., Spores, J., Notske, R., Mouser, L.T., Burroughs, R., Golden, M.S., and Lang, H.T. (1980) Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. *N. Engl. J. Med.* **303**: 897-902.

Diaz, R.J., Losito, V.A., Mao, G.D., Ford, M.K., Backx, P.H., and Wilson, G.J. (1999) Chloride channel inhibition blocks the protection of ischemic preconditioning and hypo-osmotic stress in rabbit ventricular myocardium. *Circ. Res.* **84**: 763-75.

Diraison, F., Yankah, V., Letexier, D., Dusserre, E., Jones, P., and Beylot, M. (2003) Differences in the regulation of adipose tissue and liver lipogenesis by carbohydrates in humans. *J. Lipid Res.* **44**: 846-53.

Domenech, R., Macho, P., Schwarze, H., and Sanchez, G. (2002) Exercise induces early and late myocardial preconditioning in dogs. *Cardiovasc Res.* **55**: 561-66.

Doring, H.J. and (1990) The isolated perfused heart according to langendorff technique - function - application *Physiol. Bohemoslov.* **39**: 481-504

Dos Santos, P., Kowaltowski, A.J., Laclau, M.N., Seetharaman, S., Paucek, P., Boudina, S., Thambo, J.-B., et al. (2002) Mechanisms by which opening the mitochondrial ATP- sensitive K^+ channel protects the ischemic heart. *Am J Physiol Heart Circ Physiol.* **283**: H284-95.

Dotan, Y., Lichtenberg, D., and Pinchuk, I. (2004) Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Prog. Lipid Res.* **43**: 200-27.

Downey, J.M. and Cohen, M.V. (1997) Signal transduction in ischemic preconditioning. *Adv. Exp. Med. Biol.* **430**: 39-55.

Downey, J.M. and Cohen, M.V. (2006) Reducing infarct size in the setting of acute myocardial infarction. *Prog. Cardiovasc. Dis.* **48**: 363-71.

Downey, J.M., Davis, A.M., and Cohen, M.V. (2007) Signaling pathways in ischemic preconditioning. *Heart Fail. Rev.* **12**: 181-88.

Du, X.-J., Anderson, K.E., Jacobsen, A., Woodcock, E.A., and Dart, A.M. (1995) Suppression of ventricular arrhythmias during ischemia-reperfusion by agents inhibiting ins (1,4,5) p_3 release. *Circulation.* **91**: 2712-16.

Ebrahim, S., Davey Smith, G., McCabe, C., Payne, N., Pickin, M., Sheldon, T.A., Lampe, F., et al. (1999) What role for statins? A review and economic model. *Health Technol. Assess.* **3**: i-iv, 1-91.

Elliott M. Antman, Andrew P. Selwyn, Eugene Braunwald, and Loscalzo, J. (2008), ' Ischemic heart disease', in Anthony S. Fauci, E.B., Dennis L. Kasper, Stephen L. Hauser, Dan L. Longo, J. Larry Jameson, and Joseph Loscalzo, Eds. (ed.), *Harrisons principles of internal medicine* (17 edn.; New York: McGraw-Hill Health Professions Division,).

Erdogan, H., Fadillioglu, E., Ozgocmen, S., Sogut, S., Ozyurt, B., Akyol, O., and Ardicoglu, O. (2004) Effect of fish oil supplementation on plasma oxidant/antioxidant status in rats. *Prostaglandins Leukot. Essent. Fatty Acids.* **71**: 149-52.

Fabiato, A. and Fabiato, F. (1975) Contractions induced by a calcium-triggered release of calcium from the sarcoplasmic reticulum of single skinned cardiac cells. *J. Physiol.* **249**: 469-95.

Feldman, R., Bacher, M., Campbell, N., Drover, A., and Chockalingam, A. (1998) Adherence to pharmacologic management of hypertension. *Can. J. Public Health.* **89**: I16-8.

Ferrari, R., Ceconi, C., and Guardigli, G. (2008) Pathophysiological role of heart rate: From ischaemia to left ventricular dysfunction. *Eur. Heart J. Suppl.* **10**: F7-10.

Ferrari, R., Ceconi, C., Curello, S., Alfieri, O., and Visioli, O. (1993) Myocardial damage during ischaemia and reperfusion. *Eur. Heart J.* **14 Suppl G**: 25-30.

Ferrari, R., Pepi, P., Ferrari, F., Nesta, F., Benigno, M., and Visioli, O. (1998) Metabolic derangement in ischemic heart disease and its therapeutic control. *Am. J. Cardiol.* **82**: 2K-13K.

Fickova, M., Hubert, P., Cremel, G., and Leray, C. (1998) Dietary (n-3) and (n-6) polyunsaturated fatty acids rapidly modify fatty acid composition and insulin effects in rat adipocytes. *J. Nutr.* **128**: 512-19.

Fischer, S. and Weber, P.C. (1984) Prostaglandin I3 is formed in vivo in man after dietary eicosapentaenoic acid. *Nature.* **307**: 165-8.

Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497-509.

Forbes, R.A., Steenbergen, C., and Murphy, E. (2001) Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circ Res.* **88**: 802-809.

Forman, M.B., Virmani, R., and Puett, D.W. (1990) Mechanisms and therapy of myocardial reperfusion injury. *Circulation.* **81**: 69-78.

Fox, C.S., Evans, J.C., Larson, M.G., Kannel, W.B., and Levy, D. (2004) Temporal trends in coronary heart disease mortality and sudden cardiac death from 1950 to 1999: The Framingham heart study. *Circulation.* **110**: 522-27.

Fox, K., Ford, I., Steg, P.G., Tendera, M., Robertson, M., and Ferrari, R. (2008) Heart rate as a prognostic risk factor in patients with coronary artery disease and left-ventricular systolic dysfunction (BEAUTIFUL): A subgroup analysis of a randomised controlled trial. *Lancet.* **372**: 817-21.

Frankel, E.N. (2005), *Lipid oxidation* (2nd edn., 18; California: Bridgwater, Eng: Oily Press).

Fryer, R.M., Auchampach, J.A., and Gross, G.J. (2002) Therapeutic receptor targets of ischemic preconditioning. *Cardiovasc Res.* **55**: 520-25.

Fryer, R.M., Schultz, J.E.J., Hsu, A.K., and Gross, G.J. (1999a) Importance of PKC and tyrosine kinase in single or multiple cycles of preconditioning in rat hearts. *Am J Physiol Heart Physiology.* **276**: H1229-35.

Fryer, R.M., Hsu, A.K., Eells, J.T., Nagase, H., and Gross, G.J. (1999b) Opioid-induced second window of cardioprotection: Potential role of mitochondrial KATP channels. *Circ. Res.* **84**: 846-51.

Furie, B. and Furie, B.C. (1992) Molecular and cellular biology of blood coagulation. *N. Engl. J. Med.* **326**: 800-06.

Gabbita, S.P., Robinson, K.A., Stewart, C.A., Floyd, R.A., and Hensley, K. (2000) Redox regulatory mechanisms of cellular signal transduction. *Arch. Biochem. Biophys.* **376**: 1-13.

Galinanes, M. and Hearse, D.J. (1990) Species differences in susceptibility to ischemic injury and responsiveness to myocardial protection. *Cardioscience.* **1**: 127-43.

Ganong, W.F. (2007), 'Review of medical physiology', in Kim E. Barrett, S.M.B., Scott Boitano, Heddwen Brooks (ed.), *The heart as a pump* (23e edn.; Oklahoma: McGraw-Hill Companies). http://www.accessmedicine.com/content.aspx?aID=5245513.

Gao, W.D., Liu, Y., and Marban, E. (1996) Selective effects of oxygen free radicals on excitation-contraction coupling in ventricular muscle: Implications for the mechanism of stunned myocardium. *Circulation.* **94**: 2597-604.

Gao, W.D., Atar, D., Backx, P.H., and Marban, E. (1995) Relationship between intracellular calcium and contractile force in stunned myocardium: Direct evidence for decreased myofilament Ca^{2+} responsiveness and altered diastolic function in intact ventricular muscle. *Circ. Res.* **76**: 1036-48.

Gao, W.D., Atar, D., Liu, Y., Perez, N.G., Murphy, A.M., and Marban, E. (1997) Role of troponin i proteolysis in the pathogenesis of stunned myocardium. *Circ. Res.* **80**: 393-9.

Garlid, K.D., Paucek, P., Yarov-Yarovoy, V., Sun, X., and Schindler, P.A. (1996) The mitochondrial KATP channel as a receptor for potassium channel openers. *J. Biol. Chem.* **271**: 8796-99.

Geelen, A., Brouwer, I.A., Schouten, E.G., Maan, A.C., Katan, M.B., and Zock, P.L. (2005) Effects of n-3 fatty acids from fish on premature ventricular complexes and heart rate in humans. *Am. J. Clin. Nutr.* **81**: 416-20.

Geft, I.L., Fishbein, M.C., Ninomiya, K., Hashida, J., Chaux, E., Yano, J., Y-Rit, J., et al. (1982) Intermittent brief periods of ischemia have a cumulative effect and may cause myocardial necrosis. *Circulation.* **66**: 1150-53.

Giorgi, C., Romagnoli, A., Pinton, P., and Rizzuto, R. (2008) Ca^{2+} signaling, mitochondria and cell death. *Curr Mol Med.* **8**: 119-30.

Glick, J.M., Adelman, S.J., Phillips, M.C., and Rothblat, G.H. (1983) Cellular cholesteryl ester clearance. Relationship to the physical state of cholesteryl ester inclusions. *J. Biol. Chem.* **258**: 13425-30.

Gogelein, H., Hartung, J., Englert, H.C., and Scholkens, B.A. (1998) Hmr 1883, a novel cardioselective inhibitor of the ATP-sensitive potassium channel. Part i: Effects on cardiomyocytes, coronary flow and pancreatic beta - cells. *J. Pharmacol. Exp. Ther.* **286**: 1453-64.

Goto, M., Liu, Y., Yang, X.-M., Ardell, J.L., Cohen, M.V., and Downey, J.M. (1995) Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ. Res.* **77**: 611-21.

Grimsgaard, S., Bonaa, K.H., Hansen, J.B., and Nordoy, A. (1997) Highly purified eicosapentaenoic acid and docosahexaenoic acid in humans have similar triacylglycerol-lowering effects but divergent effects on serum fatty acids. *Am. J. Clin. Nutr.* **66**: 649-59.

Grimsgaard, S., Bonaa, K.H., Hansen, J.B., and Myhre, E.S. (1998) Effects of highly purified eicosapentaenoic acid and docosahexaenoic acid on hemodynamics in humans. *Am. J. Clin. Nutr.* **68**: 52-9.

Grinwald, P.M. (1982) Calcium uptake during post-ischemic reperfusion in the isolated rat heart: Influence of extracellular sodium. *J. Mol. Cell. Cardiol.* **14**: 359-65.

Gross, G.J. (2000) The role of mitochondrial KATP channels in cardioprotection. *Basic Res. Cardiol.* **95**: 280-84.

Gross, G.J. and Fryer, R.M. (1999) Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ. Res.* **84**: 973-79.

Grynberg, A., Nalbone, G., Leonardi, J., Lafont, H., and Athias, P. (1992) Eicosapentaenoic and docosahexaenoic acids in cultured rat ventricular myocytes and hypoxia-induced alterations of phospholipase-a activity. *Mol. Cell. Biochem.* **116**: 75-8.

Gudbjarnason, S. and Hallgrimsson, J. (1976) The role of myocardial membrane lipids in the development of cardiac necrosis. *Acta Med. Scand. Suppl.* **587**: 17-27.

Gunter, T.E. and Pfeiffer, D.R. (1990) Mechanisms by which mitochondria transport calcium. *Am J Physiol Cell Physiol.* **258**: C755-86.

Guo, Y., Wu, W.-J., Qiu, Y., Tang, X.-L., Yang, Z., and Bolli, R. (1998) Demonstration of an early and a late phase of ischemic preconditioning in mice. *Am J Physiol H.* **275**: H1375-87.

Gysembergh, A., Margonari, H., Loufoua, J., Ovize, A., Andre-Fouet, X., Minaire, Y., and Ovize, M. (1998) Stretch-induced protection shares a common mechanism with ischemic preconditioning in rabbit heart. *Am J Physiol H.* **274**: H955-64.

Hagar, J.M., Hale, S.L., and Kloner, R.A. (1991) Effect of preconditioning ischemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. *Circ. Res.* **68**: 61-8.

Hallaq, H., Sellmayer, A., Smith, T.W., and Leaf, A. (1990) Protective effect of eicosapentaenoic acid on ouabain toxicity in neonatal rat cardiac myocytes. *PNAS.* **87**: 7834-38.

Halliwell, B., Gutteridge, J.M., and Cross, C.E. (1992) Free radicals, antioxidants, and human disease: Where are we now? *J. Lab. Clin. Med.* **119**: 598-620.

Hamilton, K.L., Powers, S.K., Sugiura, T., Kim, S., Lennon, S., Tumer, N., and Mehta, J.L. (2001) Short-term exercise training can improve myocardial tolerance to i/r without elevation in heat shock proteins. *Am J Physiol Heart Circ Physiol.* **281**: H1346-52.

Hansford, R.G. (1985) Relation between mitochondrial calcium transport and control of energy metabolism. *Rev. Physiol. Biochem. Pharmacol.* **102**: 1-72.

Hansford, R.G. (1987) Relation between cytosolic free Ca^{2+} concentration and the control of pyruvate dehydrogenase in isolated cardiac myocytes. *Biochem. J.* **241**: 145-51.

Hansford, R.G. (1991) Dehydrogenase activation by Ca^{2+} in cells and tissues. *J. Bioenerg. Biomembr.* **23**: 823-54.

Hansford, R.G. (1994) Physiological role of mitochondrial Ca^{2+} transport. *J. Bioenerg. Biomembr.* **26**: 495-508.

Harper, C.R. and Jacobson, T.A. (2001) The fats of life: The role of omega-3 fatty acids in the prevention of coronary heart disease. *Arch. Intern. Med.* **161**: 2185-92.

Harper, C.R. and Jacobson, T.A. (2005) Usefulness of omega-3 fatty acids and the prevention of coronary heart disease. *Am. J. Cardiol.* **96**: 1521-29.

Harris, W.S. (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans: A critical review. *J. Lipid Res.* **30**: 785-807.

Harris, W.S. (1997) n-3 fatty acids and serum lipoproteins: Human studies. *Am. J. Clin. Nutr.* **65**: 1645S-54.

Harris, W.S. and Muzio, F. (1993) Fish oil reduces postprandial triglyceride concentrations without accelerating lipid-emulsion removal rates. *Am. J. Clin. Nutr.* **58**: 68-74.

Harris, W.S., Assaad, B., and Poston, W.C. (2006a) Tissue omega-6/omega-3 fatty acid ratio and risk for coronary artery disease. *Am. J. Cardiol.* **98**: 19i-26i.

Harris, W.S., Connor, W.E., Alam, N., and Illingworth, D.R. (1988) Reduction of postprandial triglyceridemia in humans by dietary n-3 fatty acids. *J. Lipid Res.* **29**: 1451-60.

Harris, W.S., Gonzales, M., Laney, N., Sastre, A., and Borkon, A.M. (2006b) Effects of omega-3 fatty acids on heart rate in cardiac transplant recipients. *Am. J. Cardiol.* **98**: 1393-5.

Harris, W.S., Sands, S.A., Windsor, S.L., Ali, H.A., Stevens, T.L., Magalski, A., Porter, C.B., et al. (2004) Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: Correlation with erythrocytes and response to supplementation. *Circulation.* **110**: 1645-49.

Hartog, J.M., Verdouw, P.D., Klompe, M., and Lamers, J.M. (1987) Dietary mackerel oil in pigs: Effect on plasma lipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. *J. Nutr.* **117**: 1371-8.

Hassinen, I.E., Vuorinen, K.H., Ylitalo, K., and Ala-Rami, A. (1998) Role of cellular energetics in ischemia-reperfusion and ischemic preconditioning of myocardium. *Mol. Cell. Biochem.* **184**: 393-400.

He, K., Song, Y., Daviglus, M.L., Liu, K., Van Horn, L., Dyer, A.R., and Greenland, P. (2004) Accumulated evidence on fish consumption and coronary heart disease mortality: A meta-analysis of cohort studies. *Circulation.* **109**: 2705-11.

Hearse, D.J. (1988) Ischemia at the crossroads? *Cardiovasc. Drugs Ther.* **2**: 9-15.

Hearse, D.J. (1991) Reperfusion-induced injury: A possible role for oxidant stress and its manipulation. *Cardiovasc. Drugs Ther.* **5 Suppl 2**: 225-35.

Hearse, D.J. and Tosaki, A. (1987) Free radicals and reperfusion-induced arrhythmias: Protection by spin trap agent pbn in the rat heart. *Circ. Res.* **60**: 375-83.

Heineman, F.W. and Balaban, R.S. (1990) Control of mitochondrial respiration in the heart in vivo. *Annu. Rev. Physiol.* **52**: 523-42.

Helge, J.W., Wu, B.J., Willer, M., Daugaard, J.R., Storlien, L.H., and Kiens, B. (2001) Training affects muscle phospholipid fatty acid composition in humans. *J. Appl. Physiol.* **90**: 670-77.

Hellerstein, M.K., Schwarz, J.M., and Neese, R.A. (1996) Regulation of hepatic de novo lipogenesis in humans. *Annu. Rev. Nutr.* **16**: 523-57.

Herlitz, J., Karlson, B.W., and Hjalmarson, A. (1994) Ten year mortality in relation to original size of myocardial infarct: Results from the gothenburg metoprolol study. *Heart.* **71**: 238-41.

Heusch, G. (2001) Nitroglycerin and delayed preconditioning in humans: Yet another new mechanism for an old drug? *Circulation.* **103**: 2876-8.

Health Insurance Commission (HIC) Pharmaceutical Benefits Scheme statistical report. Health Insurance Commission; c2004 [cited 2009 September]. Available from: https://www.medicareaustralia.gov.au/statistics/pbs_item.shtml

Hill, M., Takano, H., Tang, X.-L., Kodani, E., Shirk, G., and Bolli, R. (2001) Nitroglycerin induces late preconditioning against myocardial infarction in conscious rabbits despite development of nitrate tolerance. *Circulation.* **104**: 694-99.

Hirafuji, M., Machida, T., Hamaue, N., and Minami, M. (2003) Cardiovascular protective effects of n-3 polyunsaturated fatty acids with special emphasis on docosahexaenoic acid. *J Pharmacol Sci.* **92**: 308-16.

Hock, C.E., Beck, L.D., Bodine, R.C., and Reibel, D.K. (1990) Influence of dietary n-3 fatty acids on myocardial ischemia and reperfusion. *Am J Physiol H.* **259**: H1518-26.

Holman, R.T. (1971), 'Essential fatty acid deficiency', *Prog. Chem. Fats other lipids* (9 edn.), 275-348.

Holmuhamedov, E.L., Wang, L., and Terzic, A. (1999) ATP-sensitive K^+ channel openers prevent Ca^{2+} overload in rat cardiac mitochondria. *J. Physiol.* **519**: 347-60.

Holmuhamedov, E.L., Jovanovic, S., Dzeja, P.P., Jovanovic, A., and Terzic, A. (1998) Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. *Am J Physiol Heart Circ Physiol.* **275**: H1567-76.

Honen, B.N., Saint, D.A., and Laver, D.R. (2003) Suppression of calcium sparks in rat ventricular myocytes and direct inhibition of sheep cardiac ryr channels by EPA, DHA and oleic acid. *J. Membr. Biol.* **196**: 95-103.

Hong, M.Y., Chapkin, R.S., Barhoumi, R., Burghardt, R.C., Turner, N.D., Henderson, C.E., Sanders, L.M., et al. (2002) Fish oil increases mitochondrial phospholipid unsaturation, upregulating reactive oxygen species and apoptosis in rat colonocytes. *Carcinogenesis.* **23**: 1919-26.

Hoshida, S., Yamashita, N., Otsu, K., and Hori, M. (2002) The importance of manganese superoxide dismutase in delayed preconditioning: Involvement of reactive oxygen species and cytokines. *Cardiovasc Res.* **55**: 495-505.

Hoshida, S., Kuzuya, T., Fuji, H., Yamashita, N., Oe, H., Hori, M., Suzuki, K., et al. (1993) Sublethal ischemia alters myocardial antioxidant activity in canine heart. *Am J Physiol Heart Circ Physiol.* **264**: H33-39.

Howe, P.R. (1997) Dietary fats and hypertension. Focus on fish oil. *Ann. N.Y. Acad. Sci.* **827**: 339-52.

Hu, F.B., Bronner, L., Willett, W.C., Stampfer, M.J., Rexrode, K.M., Albert, C.M., Hunter, D., et al. (2002) Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. *JAMA.* **287**: 1815-21.

Hu, X.-Q., Singh, N., Mukhopadhyay, D., and Akbarali, H.I. (1998) Modulation of voltage-dependent Ca^{2+} channels in rabbit colonic smooth muscle cells by c-src and focal adhesion kinase. *J. Biol. Chem.* **273**: 5337-42.

Hudgins, L.C., Hellerstein, M., Seidman, C., Neese, R., Diakun, J., and Hirsch, J. (1996) Human fatty acid synthesis is stimulated by a eucaloric low fat, high carbohydrate diet. *J. Clin. Invest.* **97**: 2081-91.

Huikuri, H.V., Castellanos, A., and Myerburg, R.J. (2001) Sudden death due to cardiac arrhythmias. *N. Engl. J. Med.* **345**: 1473-82.

Hulbert, A.J. and Else, P.L. (1999) Membranes as possible pacemakers of metabolism. *J. Theor. Biol.* **199**: 257-74.

Humphries, K.M., Yoo, Y., and Szweda, L.I. (1998) Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry (Mosc).* **37**: 552-7.

Hutter, M.M., Sievers, R.E., Barbosa, V., and Wolfe, C.L. (1994) Heat-shock protein induction in rat hearts. A direct correlation between the amount of heat-shock protein induced and the degree of myocardial protection. *Circulation.* **89**: 355-60.

Ibrahim, W., Lee, U.-S., Szabo, J., Bruckner, G., and Chow, C.K. (1999) Oxidative stress and antioxidant status in mouse kidney: Effects of dietary lipid and vitamin E plus iron. *J N Biochem.* **10**: 674-78.

Ikonomidis, J.S., Tumiati, L.C., Weisel, R.D., Mickle, D.A.G., and Li, R.-K. (1994) Preconditioning human ventricular cardiomyocytes with brief periods of simulated ischaemia. *Cardiovasc Res.* **28**: 1285-91.

Imagawa, J., Baxter, G.F., and Yellon, D.M. (1998) Myocardial protection afforded by nicorandil and ischaemic preconditioning in a rabbit infarct model in vivo. *J. Cardiovasc. Pharmacol.* **31**: 74-9.

Imagawa, J., Yellon, D.M., and Baxter, G.F. (1999) Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. *Br. J. Pharmacol.* **126**: 701-8.

Inserte, J., Garcia-Dorado, D., Ruiz-Meana, M., Solares, J., and Soler, J. (1997) The role of Na^+-H^+ exchange occurring during hypoxia in the genesis of reoxygenation-induced myocardial oedema. *J. Mol. Cell. Cardiol.* **29**: 1167-75.

Iso, H., Kobayashi, M., Ishihara, J., Sasaki, S., Okada, K., Kita, Y., Kokubo, Y., et al. (2006) Intake of fish and n3 fatty acids and risk of coronary heart disease among japanese: The japan public health center-based (jphc) study cohort i. *Circulation.* **113**: 195-202.

Jacobson, T.A. (2006) Secondary prevention of coronary artery disease with omega-3 fatty acids. *Am. J. Cardiol.* **98**: 61i-70i.

James, M.J., Gibson, R.A., and Cleland, L.G. (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production1. *Am. J. Clin. Nutr.* **71**: 343S-48.

Jennings, R.B., Sommers, H.M., Smyth, G.A., Flack, H.A., and Linn, H. (1960) Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch. Pathol.* **70**: 68-78.

Jennings, R.B., Schaper, J., Hill, M.L., Steenbergen, C., Jr., and Reimer, K.A. (1985) Effect of reperfusion late in the phase of reversible ischemic injury. Changes in cell volume, electrolytes, metabolites, and ultrastructure. *Circ. Res.* **56**: 262-78.

Jiang, W.G., Bryce, R.P., and Horrobin, D.F. (1998) Essential fatty acids: Molecular and cellular basis of their anti-cancer action and clinical implications. *Crit. Rev. Oncol. Hematol.* **27**: 179-209.

Jones, R.M., Bagchi, M., and Das, D.K. (1992) Preconditioning of heart by repeated stunning: Adaptive modification of myocardial lipid membrane. *Basic Res. Cardiol.* **87**: 527-35.

Jouven, X., Zureik, M., Desnos, M., Guerot, C., and Ducimetiere, P. (2001) Resting heart rate as a predictive risk factor for sudden death in middle-aged men. *Cardiovasc. Res.* **50**: 373-8.

Kang, X., J., and Leaf, A. (1994) Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes. *PNAS.* **91**: 9886-90.

Kang, X., J., and Leaf, A. (1995a) Prevention and termination of [beta]-adrenergic agonist-induced arrhythmias by free polyunsaturated fatty acids in neonatal rat cardiac myocytes. *Biochem. Biophys. Res. Commun.* **208**: 629-36.

Kang, X., J., and Leaf, A. (1996) Protective effects of free polyunsaturated fatty acids on arrhythmias induced by lysophosphatidylcholine or palmitoylcarnitine in neonatal rat cardiac myocytes. *Eur. J. Pharmacol.* **297**: 97-106.

Kang, X., J., and Leaf, A. (2000) Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. *Am. J. Clin. Nutr.* **71**: 202S-7S.

Kang, X., J., Xiao, Y., and Leaf, A. (1995b) Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes. *PNAS.* **92**: 3997-4001.

Kannel, W.B., Kannel, C., Paffenbarger, R.S., and Cupples, L.A. (1987) Heart rate and cardiovascular mortality: The Framingham study. *Am. Heart J.* **113**: 1489-94.

Katz, A.M. and Hecht, H.H. (1969) Editorial: The early "Pump" Failure of the ischemic heart. *Am. J. Med.* **47**: 497-502.

Kawai, Y., Fujii, H., Okada, M., Tsuchie, Y., Uchida, K., and Osawa, T. (2006) Formation of n{epsilon}-(succinyl)lysine in vivo: A novel marker for docosahexaenoic acid-derived protein modification. *J. Lipid Res.* **47**: 1386-98.

Kellogg, E.W. and Fridovich, I. (1975) Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J. Biol. Chem.* **250**: 8812-17.

Kevin, L.G., Camara, A.K.S., Riess, M.L., Novalija, E., and Stowe, D.F. (2003) Ischemic preconditioning alters real-time measure of O_2 radicals in intact hearts with ischemia and reperfusion. *Am J Physiol Heart Circ Physiol.* **284**: H566-74.

Kihara, Y. and Morgan, J.P. (1991) Intracellular calcium and ventricular fibrillation. Studies in the aequorin-loaded isovolumic ferret heart. *Circ. Res.* **68**: 1378-89.

Kihara, Y., Grossman, W., and Morgan, J.P. (1989) Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia, and reperfusion of the intact mammalian heart. *Circ. Res.* **65**: 1029-44.

King, L.M. and Opie, L.H. (1998) Glucose and glycogen utilisation in myocardial ischemia--changes in metabolism and consequences for the myocyte. *Mol. Cell. Biochem.* **180**: 3-26.

Kinoshita, I., Itoh, K., Nishida-Nakai, M., Hirota, H., Otsuji, S., and Shibata, N. (1994) Antiarrhythmic effects of eicosapentaenoic acid during myocardial infarction--enhanced cardiac microsomal (Ca^{2+}-mg^{2+}) ATPase activity. *Jpn. Circ. J.* **58**: 903-12.

Kinsella, J.E., Lokesh, B., and Stone, R.A. (1990) Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: Possible mechanisms. *Am. J. Clin. Nutr.* **52**: 1-28.

Kloner, R.A. (1993) Does reperfusion injury exist in humans? *J. Am. Coll. Cardiol.* **21**: 537-45.

Kloner, R.A. (2006) Moving preconditioning from bench to bedside. *J. Am. Coll. Cardiol.* **48**: 215-16.

Kloner, R.A. and Jennings, R.B. (2001) Consequences of brief ischemia: Stunning, preconditioning, and their clinical implications: Part 2. *Circulation.* **104**: 3158-67.

Kloner, R.A. and Rezkalla, S.H. (2006) Preconditioning, postconditioning and their application to clinical cardiology. *Cardiovasc. Res.* **70**: 297-307.

Kloner, R.A., Przyklenk, K., and Whittaker, P. (1989) Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved issues. *Circulation.* **80**: 1115-27.

Kloner, R.A., Bolli, R., Marban, E., Reinlib, L., and Braunwald, E. (1998) Medical and cellular implications of stunning, hibernation, and preconditioning: An NHLBI workshop. *Circulation.* **97**: 1848-67.

Knapp, H.R. (1997) Dietary fatty acids in human thrombosis and hemostasis. *Am. J. Clin. Nutr.* **65**: 1687S-98.

Knight, R.J., Kofoed, K.F., Schelbert, H.R., and Buxton, D.B. (1996) Inhibition of glyceraldehyde-3-phosphate dehydrogenase in post-ischaemic myocardium. *Cardiovasc Res.* **32**: 1016-23.

Koning, M.M.G., Gho, B.C.G., van Klaarwater, E., Opstal, R.L.J., Duncker, D.J., and Verdouw, P.D. (1996) Rapid ventricular pacing produces myocardial protection by nonischemic activation of $KATP^{+}$ channels. *Circulation.* **93**: 178-86.

Krause, S.M., Jacobus, W.E., and Becker, L.C. (1989) Alterations in cardiac sarcoplasmic reticulum calcium transport in the postischemic "Stunned" Myocardium. *Circ. Res.* **65**: 526-30.

Krenz, M., Baines, C.P., Heusch, G., Downey, J.M., and Cohen, M.V. (2001) Acute alcohol-induced protection against infarction in rabbit hearts: Differences from and similarities to ischemic preconditioning. *J. Mol. Cell. Cardiol.* **33**: 2015-22.

Kris-Etherton, P.M., Harris, W.S., Appel, L.J., and for the Nutrition, C. (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation.* **106**: 2747-57.

Kris-Etherton, P.M., Taylor, D.S., Yu-Poth, S., Huth, P., Moriarty, K., Fishell, V., Hargrove, R.L., et al. (2000) Polyunsaturated fatty acids in the food chain in the united states. *Am. J. Clinical Nutrition.* **71**: 179S-88.

Kristal-Boneh, E., Silber, H., Harari, G., and Froom, P. (2000) The association of resting heart rate with cardiovascular, cancer and all-cause mortality. Eight year follow-up of 3527 male israeli employees (the CORDIS study). *Eur. Heart J.* **21**: 116-24.

Kristian, T. and Siesjo, B.K. (1998) Calcium in ischemic cell death. *Stroke.* **29**: 705-18.

Kromhout, D., Bosschieter, E.B., and de Lezenne Coulander, C. (1985) The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *N. Engl. J. Med.* **312**: 1205-09.

Kureishi, Y., Kobayashi, S., Amano, M., Kimura, K., Kanaide, H., Nakano, T., Kaibuchi, K., et al. (1997) Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.* **272**: 12257-60.

Kuriyama, H., Kitamura, K., and Nabata, H. (1995) Pharmacological and physiological significance of ion channels and factors that modulate them in vascular tissues. *Pharmacol. Rev.* **47**: 387-573.

Kuzmin, A.I., Lakomkin, V.L., Kapelko, V.I., and Vassort, G. (1998) Interstitial ATP level and degradation in control and postmyocardial infarcted rats. *Am J Physiol Cell Physiol.* **275**: C766-71.

Kuzuya, T., Hoshida, S., Yamashita, N., Fuji, H., Oe, H., Hori, M., Kamada, T., et al. (1993) Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ. Res.* **72**: 1293-99.

Lakatta, E.G., Vinogradova, T.M., and Maltsev, V.A. (2008) The missing link in the mystery of normal automaticity of cardiac pacemaker cells. *Ann. N. Y. Acad. Sci.* **1123**: 41-57.

Landmark, K. (1998) [fish, fish oils, arrhythmias and sudden death]. *Tidsskr. Nor. Laegeforen.* **118**: 2328-31.

Landmark, K., Abdelnoor, M., Kilhovd, B., and Dorum, H.P. (1998a) Eating fish may reduce infarct size and the occurrence of Q wave infarcts. *Eur. J. Clin. Nutr.* **52**: 40-4.

Landmark, K., Abdelnoor, M., Urdal, P., Kilhovd, B., Dorum, H.P., Borge, N., and Refvem, H. (1998b) Use of fish oils appears to reduce infarct size as estimated from peak creatine kinase and lactate dehydrogenase activities. *Cardiology.* **89**: 94-102.

Langsjoen, P.H., Langsjoen, J.O., Langsjoen, A.M., and Lucas, L.A. (2005) Treatment of statin adverse effects with supplemental coenzyme q10 and statin drug discontinuation. *Biofactors.* **25**: 147-52.

Laser, M., Kasi, V.S., Hamawaki, M., Cooper Iv, G., Kerr, C.M., and Kuppuswamy, D. (1998) Differential activation of p70 and p85 s6 kinase isoforms during cardiac hypertrophy in the adult mammal. *J. Biol. Chem.* **273**: 24610-19.

Law, M.R., Watt, H.C., and Wald, N.J. (2002) The underlying risk of death after myocardial infarction in the absence of treatment. *Arch. Intern. Med.* **162**: 2405-10.

Lawson, C.S. and Downey, J.M. (1993) Preconditioning: State of the art myocardial protection. *Cardiovasc Res.* **27**: 542-50.

Lawson, C.S. and Hearse, D.J. (1994) Ischemic preconditioning against arrhythmias: An anti-arrhythmic or an anti-ischemic phenomenon? *Ann. N.Y. Acad. Sci.* **723**: 138-57.

Leaf, A. (2001) The electrophysiological basis for the antiarrhythmic actions of polyunsaturated fatty acids. *Eur. Heart J. Suppl.* **3**: D98-105.

Leaf, A., Kang, J.X., Xiao, Y.F., and Billman, G.E. (1999) n-3 fatty acids in the prevention of cardiac arrhythmias. *Lipids.* **34 Suppl**: S187-9.

Leaf, A., Kang, J.X., Xiao, Y.-F., and Billman, G.E. (2003) Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. *Circulation.* **107**: 2646-52.

Lee, J., Yu, B., and Herlihy, J. (1999) Modulation of cardiac mitochondrial membrane fluidity by age and calorie intake. *Free Radic. Biol. Med.* **26**: 260-65.

Leibovitz, B.E., Hu, M.L., and Tappel, A.L. (1990) Lipid peroxidation in rat tissue slices: Effect of dietary vitamin E, corn oil-lard and menhaden oil. *Lipids.* **25**: 125-9.

Leifert, W.R., Dorian, C.L., Jahangiri, A., and McMurchie, E.J. (2001) Dietary fish oil prevents asynchronous contractility and alters Ca^{2+} handling in adult rat cardiomyocytes. *J Nutr Biochem.* **12**: 365-76.

Lepage, G. and Roy, C.C. (1986) Direct transesterification of all classes of lipids in a one-step reaction. *J. Lipid Res.* **27**: 114-20.

Lepage, G., Munoz, G., Champagne, J., and Roy, C.C. (1991) Preparative steps necessary for the accurate measurement of malondialdehyde by high-performance liquid chromatography. *Anal. Biochem.* **197**: 277-83.

Lesnefsky, E.J., Tandler, B., Ye, J., Slabe, T.J., Turkaly, J., and Hoppel, C.L. (1997) Myocardial ischemia decreases oxidative phosphorylation through cytochrome oxidase in subsarcolemmal mitochondria. *Am J Physiol Heart Circ Physiol.* **273**: H1544-54.

Levitan, I.B. (1994) Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.* **56**: 193-212.

Levy, D., Kenchaiah, S., Larson, M.G., Benjamin, E.J., Kupka, M.J., Ho, K.K., Murabito, J.M., et al. (2002) Long-term trends in the incidence of and survival with heart failure. *N. Engl. J. Med.* **347**: 1397-402.

Li, G.C., Vasquez, J.A., Gallagher, K.P., and Lucchesi, B.R. (1990) Myocardial protection with preconditioning. *Circulation.* **82**: 609-19.

Li, Y., Kang, J.X., and Leaf, A. (1997) Differential effects of various eicosanoids on the production or prevention of arrhythmias in cultured neonatal rat cardiac myocytes. *Prostaglandins.* **54**: 511-30.

Li, Y.W., Whittaker, P., and Kloner, R.A. (1992) The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am. Heart J.* **123**: 346-53.

Lichtenstein, A.H., Appel, L.J., Brands, M., Carnethon, M., Daniels, S., Franch, H.A., Franklin, B., et al. (2006) Diet and lifestyle recommendations revision 2006: A scientific statement from the american heart association nutrition committee. *Circulation.* **114**: 82-96.

Liu, G.S., Thornton, J., Van Winkle, D.M., Stanley, A.W., Olsson, R.A., and Downey, J.M. (1991) Protection against infarction afforded by preconditioning is mediated by a1 adenosine receptors in rabbit heart. *Circulation.* **84**: 350-6.

Liu, G.S., Richards, S.C., Olsson, R.A., Mullane, K., Walsh, R.S., and Downey, J.M. (1994) Evidence that the adenosine A3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res.* **28**: 1057-61.

Liu, Y. and Downey, J.M. (1992) Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol heart Circ Physiol.* **263**: H1107-12.

Liu, Y., Sato, T., O'Rourke, B., and Marban, E. (1998) Mitochondrial ATP-dependent potassium channels: Novel effectors of cardioprotection? *Circulation.* **97**: 2463-69.

Liu, Z., Kastis, G.A., Stevenson, G.D., Barrett, H.H., Furenlid, L.R., Kupinski, M.A., Patton, D.D., et al. (2002) Quantitative analysis of acute myocardial infarct in rat hearts with ischemia-reperfusion using a high-resolution stationary spect system. *J. Nucl. Med.* **43**: 933-39.

Llanillo, M., Sanchez-Yague, J., Checa, A., Martin-Valmaseda, E.M., and Felipe, A. (1995) Phospholipid and fatty acid composition in stored sheep erythrocytes of different densities. *Exp. Hematol.* **23**: 258-64.

Loeliger, E.A., Hensen, A., Kroes, F., van Dijk, L.M., Fekkes, N., de Jonge, H., and Hemker, H.C. (1967) A double-blind trial of long-term anticoagulant treatment after myocardial infarction. *Acta Med. Scand.* **182**: 549-66.

Lorenz, R.L., Schacky, C.V., Weber, M., Meister, W., Kotzur, J., Reichardt, B., Theisen, K., et al. (1984) Improved aortocoronary bypass patency by low-

dose aspirin (100 mg daily). Effects on platelet aggregation and thromboxane formation. *Lancet.* **1**: 1261-4.

Loscalzo, J. and Vita, J.A. (1994) Ischemia, hyperemia, exercise, and nitric oxide. Complex physiology and complex molecular adaptations. *Circulation.* **90**: 2556-59.

Lucas, D.T. and Szweda, L.I. (1998) Cardiac reperfusion injury: Aging, lipid peroxidation, and mitochondrial dysfunction. *Proc. Natl. Acad. Sci. U. S. A.* **95**: 510-14.

Lundberg, K.C. and Szweda, L.I. (2006) Preconditioning prevents loss in mitochondrial function and release of cytochrome c during prolonged cardiac ischemia/reperfusion. *Arch. Biochem. Biophys.* **453**: 130-4.

Luostarinen, R., Wallin, R., and Saldeen, T. (1997) Dietary (n-3) fatty acids increase superoxide dismutase activity and decrease thromboxane production in the rat heart. *Nutr Res.* **17**: 163-75.

Mackay, J. and Mensah, G.A. (2004) 'The atlas of heart disease and stroke', in Hayley Ann. (Ed), *Cardiovascular disease* (1e edn.; Geneva: WHO). [cited March, 2010] http://www.who.int/cardiovascular_diseases/resources/atlas/en/

Majno, G. and Joris, I. (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* **146**: 3-15.

Marber, M.S., Latchman, D.S., Walker, J.M., and Yellon, D.M. (1993) Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation.* **88**: 1264-72.

Marchioli, Roberto., Barzi, F., Bomba, E., and Chieffo, C. (2002) Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: Time-course analysis of the results of the gruppo italiano per lo studio della sopravvivenza nell'infarto miocardico (GISSI)-prevenzione. *Circulation.* **105**: 1897-903.

Marchioli, R., Schweiger, C., Tavazzi, L., and Valagussa, F. (2001) Efficacy of (n-3) polyunsaturated fatty acids after myocardial infarction: Results of GISSI-prevenzione trial. Gruppo italiano per lo studio della sopravvivenza nell'infarto miocardico. *Lipids.* **36 Suppl**: S119-26.

Marchioli, R., Levantesi, G., Macchia, A., Maggioni, A.P., Marfisi, R.M., Silletta, M.G., Tavazzi, L., et al. (2005) Antiarrhythmic mechanisms of (n-3) PUFA and the results of the GISSI-prevenzione trial. *J. Membr. Biol.* **206**: 117-28.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., et al. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.* **15**: 2208-16.

Matthan, N.R., Jordan, H., Chung, M., Lichtenstein, A.H., Lathrop, D.A., and Lau, J. (2005) A systematic review and meta-analysis of the impact of omega-3 fatty acids on selected arrhythmia outcomes in animal models. *Metabolism.* **54**: 1557-65.

McDevitt, R.M., Bott, S.J., Harding, M., Coward, W.A., Bluck, L.J., and Prentice, A.M. (2001) De novo lipogenesis during controlled overfeeding with sucrose or glucose in lean and obese women. *Am. J. Clin. Nutr.* **74**: 737-46.

McLennan, P.L. (1993) Relative effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on cardiac arrhythmias in rats. *Am. J. Clin. Nutr.* **57**: 207-12.

McLennan, P.L. (2001) Myocardial membrane fatty acids and the antiarrhythmic actions of dietary fish oil in animal models. *Lipids.* **36 Suppl**: S111-4.

McLennan, P.L. (2004) Omega-3 polyunsaturated fatty acid prevention of cardiac arrhythmia and sudden death: Cellular or circulating? *Curr Top Nuraceut Res.* **2**: 101-11.

McLennan, P.L. and Dallimore, J.A. (1995) Dietary canola oil modifies myocardial fatty acids and inhibits cardiac arrhythmias in rats. *J. Nutr.* **125**: 1003-9.

McLennan, P.L. and Abeywardena, M.Y. (2005) Membrane basis for fish oil effects on the heart: Linking natural hibernators to prevention of human sudden cardiac death. *J. Membr. Biol.* **206**: 85-102.

McLennan, P.L., Abeywardena, M.Y., and Charnock, J.S. (1985) Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. *Can. J. Physiol. Pharmacol.* **63**: 1411-7.

McLennan, P.L., Abeywardena, M.Y., and Charnock, J.S. (1988) Dietary fish oil prevents ventricular fibrillation following coronary artery occlusion and reperfusion. *Am. Heart J.* **116**: 709-17.

McLennan, P.L., Bridle, T.M., Abeywardena, M.Y., and Charnock, J.S. (1992a) Dietary lipid modulation of ventricular fibrillation threshold in the marmoset monkey. *Am. Heart J.* **123**: 1555-61.

McLennan, P.L., Bridle, T.M., Abeywardena, M.Y., and Charnock, J.S. (1993) Comparative efficacy of n-3 and n-6 polyunsaturated fatty acids in modulating ventricular fibrillation threshold in marmoset monkeys. *Am. J. Clin. Nutr.* **58**: 666-9.

McLennan, P.L., Barnden, L.R., Bridle, T.M., Abeywardena, M.Y., and Charnock, J.S. (1992b) Dietary fat modulation of left ventricular ejection fraction in the marmoset due to enhanced filling. *Cardiovasc. Res.* **26**: 871-7.

McLennan, P.L., Howe, P., Abeywardena, M., Muggli, R., Raederstorff, D., Mano, M., Rayner, T., et al. (1996) The cardiovascular protective role of docosahexaenoic acid. *Eur. J. Pharmacol.* **300**: 83-9.

McLennan, P.L., Owen, A.J., Slee, E.L., and Theiss, M.L. (2007) Myocardial function, ischaemia and n-3 polyunsaturated fatty acids: A membrane basis. *Journal of Cardiovascular Medicine.* **8**: S15-S18

Meissner, M.D., Akhtar, M., and Lehmann, M.H. (1991) Nonischemic sudden tachyarrhythmic death in atherosclerotic heart disease. *Circulation.* **84**: 905-12.

Meller, N., Altman, A., and Isakov, N. (1998) New perspectives on PKC{theta}, a member of the novel subfamily of protein kinase c. *Stem Cells.* **16**: 178-92.

Mellor, H. and Parker, P.J. (1998) The extended protein kinase c superfamily. *Biochem. J.* **332 (Pt 2)**: 281-92.

Metcalf, R.G., James, M.J., Gibson, R.A., Edwards, J.R.M., Stubberfield, J., Stuklis, R., Roberts-Thomson, K., et al. (2007) Effects of fish-oil supplementation on myocardial fatty acids in humans. *Am. J. Clin. Nutr.* **85**: 1222-28.

Mettauer, B., Zoll, J., Garnier, A., and Ventura-Clapier, R. (2006) Heart failure: A model of cardiac and skeletal muscle energetic failure. *Pflugers Arch.* **452**: 653-66.

Mizushima, S., Moriguchi, E.H., Ishikawa, P., Hekman, P., Nara, Y., Mimura, G., Moriguchi, Y., et al. (1997) Fish intake and cardiovascular risk among middle-aged japanese in japan and brazil. *J. Cardiovasc. Risk.* **4**: 191-9.

Monnet, X., Ghaleh, B., Lucats, L., Colin, P., Zini, R., Hittinger, L., and Berdeaux, A. (2006) Phenotypic adaptation of the late preconditioned heart: Myocardial oxygen consumption is reduced. *Cardiovasc. Res.* **70**: 391-98.

Moore, K.J. and Freeman, M.W. (2006) Scavenger receptors in atherosclerosis: Beyond lipid uptake. *Arterioscler. Thromb. Vasc. Biol.* **26**: 1702-11.

Moreadith, R.W. and Collen, D. (2003) Clinical development of pegylated recombinant staphylokinase (peg-sak) for bolus thrombolytic treatment of patients with acute myocardial infarction. *ADV DRUG Deliv REV.* **55**: 1337-45.

Mori, T.A., Beilin, L.J., Burke, V., Morris, J., and Ritchie, J. (1997) Interactions between dietary fat, fish, and fish oils and their effects on platelet function in men at risk of cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* **17**: 279-86.

Mori, T.A., Watts, G.F., Burke, V., Hilme, E., Puddey, I.B., and Beilin, L.J. (2000) Differential effects of eicosapentaenoic acid and docosahexaenoic acid on vascular reactivity of the forearm microcirculation in hyperlipidemic, overweight men. *Circulation.* **102**: 1264-69.

Morris, M.C., Sacks, F., and Rosner, B. (1993) Does fish oil lower blood pressure? A meta-analysis of controlled trials. *Circulation.* **88**: 523-33.

Morris, S.D. and Yellon, D.M. (1997) Angiotensin-converting enzyme inhibitors potentiate preconditioning through bradykinin b2 receptor activation in human heart. *J. Am. Coll. Cardiol.* **29**: 1599-606.

Mouri, K., Ikesu, H., Esaka, T., and Igarashi, O. (1984) The influence of marine oil intake upon levels of lipids, alpha-tocopherol and lipid peroxidation in serum and liver of rats. *J. Nutr. Sci. Vitaminol. (Tokyo).* **30**: 307-18.

Mozaffarian, D. (2008) Fish and n-3 fatty acids for the prevention of fatal coronary heart disease and sudden cardiac death. *Am. J. Clin. Nutr.* **87**: 1991S-96.

Mozaffarian, D., Psaty, B.M., and Romm, E.B. (2004) Fish intake and risk of incident atrial fibrillation. *ACC Current Journal Review.* **13**: 12-1529.

Mozaffarian, D., Stein, P.K., Prineas, R.J., and Siscovick, D.S. (2008) Dietary fish and {omega}-3 fatty acid consumption and heart rate variability in us adults. *Circulation.* **117**: 1130-37.

Mozaffarian, D., Bryson, C.L., Lemaitre, R.N., Burke, G.L., and Siscovick, D.S. (2005a) Fish intake and risk of incident heart failure. *J. Am. Coll. Cardiol.* **45**: 2015-21.

Mozaffarian, D., Lemaitre, R.N., Kuller, L.H., Burke, G.L., Tracy, R.P., and Siscovick, D.S. (2003) Cardiac benefits of fish consumption may depend

on the type of fish meal consumed: The cardiovascular health study. *Circulation.* **107**: 1372-77.

Mozaffarian, D., Geelen, A., Brouwer, I.A., Geleijnse, J.M., Zock, P.L., and Katan, M.B. (2005b) Effect of fish oil on heart rate in humans: A meta-analysis of randomized controlled trials. *Circulation.* **112**: 1945-52.

Mozaffarian, D., Longstreth, W.T., Jr., Lemaitre, R.N., Manolio, T.A., Kuller, L.H., Burke, G.L., and Siscovick, D.S. (2005c) Fish consumption and stroke risk in elderly individuals: The cardiovascular health study. *Arch. Intern. Med.* **165**: 200-06.

Mozaffarian, D., Ascherio, A., Hu, F.B., Stampfer, M.J., Willett, W.C., Siscovick, D.S., and Rimm, E.B. (2005d) Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation.* **111**: 157-64.

Mubagwa, K., Kaplan, P., and Flameng, W. (1997) The effects of ryanodine on calcium uptake by the sarcoplasmic reticulum of ischemic and reperfused rat myocardium. *Fundam. Clin. Pharmacol.* **11**: 315-21.

Murphy, E., Glasgow, W., Fralix, T., and Steenbergen, C. (1995) Role of lipoxygenase metabolites in ischemic preconditioning. *Circ. Res.* **76**: 457-67.

Murray, C.J. and Lopez, A.D. (1997) Alternative projections of mortality and disability by cause 1990-2020: Global burden of disease study. *Lancet.* **349**: 1498-504.

Murry, C.E., Jennings, R.B., and Reimer, K.A. (1986) Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation.* **74**: 1124-36.

Murry CE, J.R. and KA, R. (1994), *What is ischemic preconditioning?*, ed. K, P. (Ischemic preconditioning: The concept of endogenous cardioprotection; Boston: Kluwer Academic Publisher) 3-17.

Myerburg and Robert, J. (2005), *A textbook of cardiovascular medicine* (7th edition edn.; Philadelphia: WB Saunders).

Myerburg, J., R., Kessler, K.M., and Castellanos, A. (1993) Sudden cardiac death: Epidemiology, transient risk, and intervention assessment. *Ann. Intern. Med.* **119**: 1187-97.

Nair, S.S., Leitch, J., and Garg, M.L. (2000) Suppression of inositol phosphate release by cardiac myocytes isolated from fish oil-fed pigs. *Mol. Cell. Biochem.* **215**: 57-64.

Nair, S.S., Leitch, J.W., Falconer, J., and Garg, M.L. (1997) Prevention of cardiac arrhythmia by dietary (n-3) polyunsaturated fatty acids and their mechanism of action. *J. Nutr.* **127**: 383-93.

Nalbone, G., Leonardi, J., Termine, E., Portugal, H., Lechene, P., Pauli, A.M., and Lafont, H. (1989) Effects of fish oil, corn oil and lard diets on lipid peroxidation status and glutathione peroxidase activities in rat heart. *Lipids.* **24**: 179-86.

Nalbone, G., Termine, E., Leonardi, J., Portugal, H., Lechene, P., Calaf, R., Lafont, R., et al. (1988) Effect of dietary salmon oil feeding on rat heart lipid status. *J. Nutr.* **118**: 809-17.

NCEP (1993) Summary of the second report of the National cholesterol education program (ncep) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel ii). *JAMA.* **269**: 3015-23.

Nelson, G.J., Schmidt, P.C., and Corash, L. (1991) The effect of a salmon diet on blood clotting, platelet aggregation and fatty acids in normal adult men. *Lipids.* **26**: 87-96.

Nelson, R.R., Gobel, F.L., Jorgensen, C.R., Wang, K., Wang, Y., and Taylor, H.L. (1974) Hemodynamic predictors of myocardial oxygen consumption during static and dynamic exercise. *Circulation.* **50**: 1179-89.

Nestel, P.J. (1986) Fish oil attenuates the cholesterol induced rise in lipoprotein cholesterol. *Am. J. Clin. Nutr.* **43**: 752-57.

NHMRC (2004), Australian code of practice for the care and use of animals for scientific purposes,7th ed., (Canberra: National Health and Medical Research Council), 84.

Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase c. *Science.* **258**: 607-14.

Nordoy, A., Hatcher, L.F., Ullmann, D.L., and Connor, W.E. (1993) Individual effects of dietary saturated fatty acids and fish oil on plasma lipids and lipoproteins in normal men. *Am. J. Clinical Nutrition.* **57**: 634-39.

Novak, E.M. and Innis, S.M. (2006) Decreasing the dietary n-6/n-3 fatty acid ratio uniquely increases eicosapentaenic acid in heart membrane phospholipids with no effect on glut 4 expression in piglets. *FASEB J.* **20**: A1028 (abstract).

Nulton-Persson, A.C., Starke, D.W., Mieyal, J.J., and Szweda, L.I. (2003) Reversible inactivation of alpha-ketoglutarate dehydrogenase in response to alterations in the mitochondrial glutathione status. *Biochemistry (Mosc).* **42**: 4235-42.

Nyby, M.D., Hori, M.T., Ormsby, B., Gabrielian, A., and Tuck, M.L. (2003) Eicosapentaenoic acid inhibits Ca^{2+} mobilization and PKC activity in vascular smooth muscle cells. *Am. J. Hypertens.* **16**: 708-14.

O'Brien, J.S. and Sampson, E.L. (1965) Lipid composition of the normal human brain: Gray matter, white matter, and myelin. *J. Lipid Res.* **6**: 537-44.

O'Neill, S.C., Perez, M.R., Hammond, K.E., Sheader, E.A., and Negretti, N. (2002) Direct and indirect modulation of rat cardiac sarcoplasmic reticulum function by n-3 polyunsaturated fatty acids. *J. Physiol.* **538**: 179-84.

Oberley, L.W., St Clair, D.K., Autor, A.P., and Oberley, T.D. (1987) Increase in manganese superoxide dismutase activity in the mouse heart after x-irradiation. *Arch. Biochem. Biophys.* **254**: 69-80.

Oostenbrug, G.S., Mensink, R.P., and Hornstra, G. (1994) Effects of fish oil and vitamin E supplementation on copper-catalysed oxidation of human low density lipoprotein in vitro. *Eur. J. Clin. Nutr.* **48**: 895-8.

Opie, L.H. (2004), *Heart physiology from cell to circulation* (Fourth edn.; Philadelphia: Lippincott Williams&Wilkens).

Otani, H. (2008) Ischemic preconditioning: From molecular mechanisms to therapeutic opportunities. *Antioxid Redox Signal.* **10**: 207-47.

Ovize, M., Kloner, R.A., and Przyklenk, K. (1994) Stretch preconditions canine myocardium. *Am J Physiol Heart Circ Physiol.* **266**: H137-46.

Owen, A.J., Peter-Przyborowska, B.A., Hoy, A.J., and McLennan, P.L. (2004) Dietary fish oil dose- and time-response effects on cardiac phospholipid fatty acid composition. *Lipids.* **39**: 955-61.

Page, L.B. (1979), *Hypertension and atherosclerosis in primitive and acculturating societies*, ed. Hunt, J.C. (Hypertension update, 1; Lyndhurst,NJ: Health Learning Systems) 1-12.

Pain, T., Yang, X.-M., Critz, S.D., Yue, Y., Nakano, A., Liu, G.S., Heusch, G., et al. (2000) Opening of mitochondrial KATP channels triggers the preconditioned state by generating free radicals. *Circ. Res.* **87**: 460-66.

Panagiotakos, D.B., Pitsavos, C., Zampelas, A., Chrysohoou, C., Griffin, B.A., Stefanadis, C., and Toutouzas, P. (2005) Fish consumption and the risk of developing acute coronary syndromes: The CARDIO 2000 study. *Int. J. Cardiol.* **102**: 403-09.

Parent De Curzon, O., Ghaleh, B., Tissier, R., Giudicelli, J.-F., Hittinger, L., and Berdeaux, A. (2001) Myocardial stunning in exercise-induced ischemia in dogs: Lack of late preconditioning. *Am J Physiol Heart Circ Physiol.* **280**: H302-10.

Parks, J.S. and Bullock, B.C. (1987) Effect of fish oil versus lard diets on the chemical and physical properties of low density lipoproteins of nonhuman primates. *J. Lipid Res.* **28**: 173-82.

Parsons, J.T. and Parsons, S.J. (1997) Src family protein tyrosine kinases: Cooperating with growth factor and adhesion signaling pathways. *Curr. Opin. Cell Biol.* **9**: 187-92.

Pasantes-Morales, H., Lezama, R.A., Ramos-Mandujano, G., and Tuz, K.L. (2006) Mechanisms of cell volume regulation in hypo-osmolality. *Am. J. Med.* **119**: S4-S11.

Pawlosky, R.J., Hibbeln, J.R., Novotny, J.A., and Salem, N., Jr. (2001) Physiological compartmental analysis of {alpha}-linolenic acid metabolism in adult humans. *J. Lipid Res.* **42**: 1257-65.

Pepe and McLennan, P.L. (1996) Dietary fish oil confers direct antiarrhythmic properties on the myocardium of rats. *J. Nutr.* **126**: 34-42.

Pepe, S. (2007) Dietary polyunsaturated fatty acids and age-related membrane changes in the heart. *Ann. N. Y. Acad. Sci.* **1114**: 381-8.

Pepe, S. and McLennan, P.L. (2002) Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function. *Circulation.* **105**: 2303-08.

Pepe, S. and McLennan, P.L. (2007) (n-3) long chain PUFA dose-dependently increase oxygen utilization efficiency and inhibit arrhythmias after saturated fat feeding in rats. *J. Nutr.*137.

Pepe, S., Tsuchiya, N., Lakatta, E.G., and Hansford, R.G. (1999) PUFA and aging modulate cardiac mitochondrial membrane lipid composition and Ca^{2+} activation of pdh. *Am. J. Physiol.* **276**: H149-58.

Perez-Reyes, E. (2003) Molecular physiology of low-voltage-activated T-type calcium channels. *Physiol. Rev.* **83**: 117-61.

Phillipson, B.E., Rothrock, D.W., Connor, W.E., Harris, W.S., and Illingworth, D.R. (1985) Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in patients with hypertriglyceridemia. *N. Engl. J. Med.* **312**: 1210-16.

Pinkus, R., Weiner, L.M., and Daniel, V. (1996) Role of oxidants and antioxidants in the induction of AP-1, NF-kappa B, and glutathione s-transferase gene expression. *J. Biol. Chem.* **271**: 13422-29.

Pinton, P., Pozzan, T., and Rizzuto, R. (1998) The golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca^{2+} store, with functional properties distinct from those of the endoplasmic reticulum. *EMBO J.* **17**: 5298-308.

Piper, H.M. and Garcia-Dorado, D. (1999) Prime causes of rapid cardiomyocyte death during reperfusion. *Ann. Thorac. Surg.* **68**: 1913-19.

Pogwizd, S.M. and Bers, D.M. (2004) Cellular basis of triggered arrhythmias in heart failure. *Trends Cardiovasc. Med.* **14**: 61-66.

Poston, J.M. and Parenteau, G.L. (1992) Biochemical effects of ischemia on isolated, perfused rat heart tissues. *Arch. Biochem. Biophys.* **295**: 35-41.

Poulos, A., Darin-Bennett, A., and White, I.G. (1973) The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. **46**: 541-49.

Powers, S.K. and Lennon, S.L. (1999) Analysis of cellular responses to free radicals: Focus on exercise and skeletal muscle. *Proc. Nutr. Soc.* **58**: 1025-33.

Powers, S.K., Criswell, D., Lawler, J., Martin, D., Lieu, F.K., Ji, L.L., and Herb, R.A. (1993) Rigorous exercise training increases superoxide dismutase activity in ventricular myocardium. *Am J Physiol Heart Circ Physiol.* **265**: H2094-98.

Praticò, D. (2002) Lipid peroxidation and the aging process. *Sci. Aging Knowl. Environ.* **2002**: 1-4.

Przyklenk, K. and Kloner, R.A. (1998) Ischemic preconditioning: Exploring the paradox. *Prog. Cardiovasc. Dis.* **40**: 517-47.

Psota, T.L., Gebauer, S.K., and Kris-Etherton, P. (2006) Dietary omega-3 fatty acid intake and cardiovascular risk. *Am. J. Cardiol.* **98**: 3i-18i.

Qiu, Y., Tang, X.-L., Park, S.-W., Sun, J.-Z., Kalya, A., and Bolli, R. (1997) The early and late phases of ischemic preconditioning: A comparative analysis of their effects on infarct size, myocardial stunning, and arrhythmias in conscious pigs undergoing a 40-minute coronary occlusion. *Circ. Res.* **80**: 730-42.

Radak, Z. (2000), *Free radicals in exercise and aging* (Human kinetics; USA: Champaign).

Raz, A., Kamin-Belsky, N., Przedecki, F., and Obukowicz, M.G. (1997) Fish oil inhibits [delta]6 desaturase activity in vivo: Utility in a dietary paradigm to obtain mice depleted of arachidonic acid. *J N Biochem.* **8**: 558-65.

Reducing risks, promoting healthy life. World Health Organization; c2002 [cited 2009 September 14]. Available from http://www.who.int/whr/2002/en/

Reeves, P.G. (1997) Components of the AIN-93 diets as improvements in the ain-76a diet. *J. Nutr.* **127**: 838S.

Reiffel, J.A. and McDonald, A. (2006) Antiarrhythmic effects of omega-3 fatty acids. *Am. J. Cardiol.* **98**: 50i-60i.

Rhee, S.G. (1999) Redox signaling: Hydrogen peroxide as intracellular messenger. *Exp. Mol. Med.* **31**: 53-9.

Richard, V.J., Murry, C.E., Jennings, R.B., and Reimer, K.A. (1988) Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs. *Circulation.* **78**: 473-80.

Rizvi, A., Tang, X.-L., Qiu, Y., Xuan, Y.-T., Takano, H., Jadoon, A.K., and Bolli, R. (1999) Increased protein synthesis is necessary for the development of late preconditioning against myocardial stunning. *Am J Physiol Heart Circ Physiol.* **277**: H874-84.

Robinson, J.G. and Stone, N.J. (2006) Antiatherosclerotic and antithrombotic effects of omega-3 fatty acids. *Am. J. Cardiol.* **98**: 39i-49i.

Rocquelin, G., Guenot, L., Astorg, P.O., and David, M. (1989) Phospholipid content and fatty acid composition of human heart. *Lipids.* **24**: 775-80.

Rocquelin, G., Guenot, L., Justrabo, E., Grynberg, A., and David, M. (1985) Fatty acid composition of human heart phospholipids: Data from 53 biopsy specimens. *J. Mol. Cell. Cardiol.* **17**: 769-73.

Rousseau-Ralliard, D., Moreau, D., Guilland, J.C., Raederstorff, D., and Grynberg, A. (2009) Docosahexaenoic acid, but not eicosapentaenoic acid, lowers ambulatory blood pressure and shortens interval QT in spontaneously hypertensive rats in vivo. *Prostaglandins Leukot. Essent. Fatty Acids.* **80:** 269-277

Rousseau, D., Helies-Toussaint, C., Raederstorff, D., Moreau, D., and Grynberg, A. (2001) Dietary n-3 polyunsaturated fatty acids affect the development of renovascular hypertension in rats. *Mol. Cell. Biochem.* **225**: 109-19.

Rudiger, H.A., Graf, R., and Clavien, P.-A. (2003) Sub-lethal oxidative stress triggers the protective effects of ischemic preconditioning in the mouse liver. *J. Hepatol.* **39**: 972-77.

Ruiz-Gutierrez, V., Vazquez, C.M., and Santa-Maria, C. (2001) Liver lipid composition and antioxidant enzyme activities of spontaneously hypertensive rats after ingestion of dietary fats (fish, olive and high-oleic sunflower oils). *Biosci. Rep.* **21**: 271-85.

Ruiz-Gutierrez, V., Perez-Espinosa, A., Vazquez, C.M., and Santa-Maria, C. (1999) Effects of dietary fats (fish, olive and high-oleic-acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. *Br. J. Nutr.* **82**: 233-41.

Rustan, A.C. (2005) Fatty acids: Structures and properties. In: *Encyclopedia of Life Sciences.* John Wiley & Sons, Ltd: Chichester http://www.els.net.ezproxy.uow.edu.au/ [doi:10.1038/npg.els.0006101].

Sacheck, J.M. and Blumberg, J.B. (2001) Role of vitamin E and oxidative stress in exercise. *Nutrition.* **17**: 809-14.

Sadek, H.A., Humphries, K.M., Szweda, P.A., and Szweda, L.I. (2002) Selective inactivation of redox-sensitive mitochondrial enzymes during cardiac reperfusion. *Arch. Biochem. Biophys.* **406**: 222-8.

Sah, V.P., Hoshijima, M., Chien, K.R., and Brown, J.H. (1996) Rho is required for galpha q and alpha 1-adrenergic receptor signaling in cardiomyocytes. Dissociation of ras and Rho pathways. *J. Biol. Chem.* **271**: 31185-90.

Saito, M. and Kubo, K. (2003) Relationship between tissue lipid peroxidation and peroxidizability index after alpha-linolenic, eicosapentaenoic, or docosahexaenoic acid intake in rats. *Br. J. Nutr.* **89**: 19-28.

Salem, N., Shingu, T., Kim, H.Y., Hullin, F., Bougnoux, P., and Karanian, J.W. (1988) Specialization in membrane structure and metabolism with respect to polyunsaturated lipids. *Prog. Clin. Biol. Res.* **282**: 319-33.

Salomaa, V., Ketonen, M., Koukkunen, H., Immonen-Raiha, P., Jerkkola, T., Karja-Koskenkari, P., Mahonen, M., et al. (2003) Decline in out-of-hospital coronary heart disease deaths has contributed the main part to the overall decline in coronary heart disease mortality rates among persons 35 to 64 years of age in finland: The FINAMI study. *Circulation.* **108**: 691-96.

Sato, T., Sasaki, N., Seharaseyon, J., O'Rourke, B., and Marban, E. (2000) Selective pharmacological agents implicate mitochondrial but not sarcolemmal KATP channels in ischemic cardioprotection. *Circulation.* **101**: 2418-23.

Satoh, Y. and Shikama, K. (1981) Autoxidation of oxymyoglobin. A nucleophilic displacement mechanism. *J. Biol. Chem.* **256**: 10272-75.

Saurin, A.T., Martin, J.L., Heads, R.J., Foley, C., Mockridge, J.W., Wright, M.J., Wang, Y., et al. (2000) The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. *FASEB J.* **14**: 2237-46.

Schaffer, W.A. and Cobb, L.A. (1975) Recurrent ventricular fibrillation and modes of death in survivors of out-of-hospital ventricular fibrillation. *N. Engl. J. Med.* **293**: 259-62.

Schectman, G., Hiatt, J., and Hartz, A. (1993) Evaluation of the effectiveness of lipid-lowering therapy (bile acid sequestrants, niacin, psyllium and

lovastatin) for treating hypercholesterolemia in veterans. *Am. J. Cardiol.* **71**: 759-65.

Schneider, S., Chen, W., Hou, J., Steenbergen, C., and Murphy, E. (2001) Inhibition of p38 mapk {alpha}/{beta} reduces ischemic injury and does not block protective effects of preconditioning. *Am J Physiol Heart Circ Physiol.* **280**: H499-508.

Schott, R.J., Rohmann, S., Braun, E.R., and Schaper, W. (1990) Ischemic preconditioning reduces infarct size in swine myocardium. *Circ. Res.* **66**: 1133-42.

Schultz, J., J.E., Hsu, A.K., and Gross, G.J. (1996) Morphine mimics the cardioprotective effect of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. *Circ. Res.* **78**: 1100-04.

Schultz, E., J., Rose, E., Yao, Z., and Gross, G.J. (1995) Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. *Am J Physiol Heart Circ Physiol.* **268**: H2157-61.

Schultz, J.E.J., Hsu, A.K., and Gross, G.J. (1998) Ischemic preconditioning in the intact rat heart is mediated by {delta}1- but not μ- or {kappa}-opioid receptors. *Circulation.* **97**: 1282-89.

Schulz, R., Post, H., Vahlhaus, C., and Heusch, G. (1998) Ischemic preconditioning in pigs: A graded phenomenon: Its relation to adenosine and bradykinin. *Circulation.* **98**: 1022-29.

Seevaratnam, R., Patel, B.P., and Hamadeh, M.J. (2009) Comparison of total protein concentration in skeletal muscle as measured by the bradford and lowry assays. *J. Biochem.* **145**: 791-797.

Sexton, P.T., Sinclair, A.J., O'Dea, K., Sanigorski, A.J., and Walsh, J. (1995) The relationship between linoleic acid level in serum, adipose tissue and myocardium in humans. *Asia Pac J Clin Nutr.* **4:** 314-318

Shiki, K. and Hearse, D.J. (1987) Preconditioning of ischemic myocardium: Reperfusion-induced arrhythmias. *Am J Physiol Heart Circ Physiol.* **253**: H1470-76.

Shinmura, K., Xuan, Y.-T., Tang, X.-L., Kodani, E., Han, H., Zhu, Y., and Bolli, R. (2002) Inducible nitric oxide synthase modulates cyclooxygenase-2 activity in the heart of conscious rabbits during the late phase of ischemic preconditioning. *Circ. Res.* **90**: 602-08.

Shinmura, K., Tang, X.-L., Wang, Y., Xuan, Y.-T., Liu, S.-Q., Takano, H., Bhatnagar, A., et al. (2000) Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *PNAS.* **97**: 10197-202.

Shryock, J.C., Snowdy, S., Baraldi, P.G., Cacciari, B., Spalluto, G., Monopoli, A., Ongini, E., et al. (1998) A2A-adenosine receptor reserve for coronary vasodilation. *Circulation.* **98**: 711-18.

Siddiqui, R.A., Harvey, K.A., and Zaloga, G.P. (2008) Modulation of enzymatic activities by n-3 polyunsaturated fatty acids to support cardiovascular health. *J Nutr Biochem.* **19**: 417-37.

Siddiqui, R.A., Shaikh, S.R., Kovacs, R., Stillwell, W., and Zaloga, G. (2004) Inhibition of phenylephrine-induced cardiac hypertrophy by docosahexaenoic acid. *J. Cell. Biochem.* **92**: 1141-59.

Simes, R.J., Topol, E.J., Holmes, D.R., Jr., White, H.D., Rutsch, W.R., Vahanian, A., Simoons, M.L., et al. (1995) Link between the angiographic substudy and mortality outcomes in a large randomized trial of myocardial reperfusion: Importance of early and complete infarct artery reperfusion. *Circulation.* **91**: 1923-28.

Simkhovich, B.Z., Przyklenk, K., and Kloner, R.A. (1998) Role of protein kinase c as a cellular mediator of ischemic preconditioning: A critical review. *Cardiovasc Res.* **40**: 9-22.

Simons, A., L., Hickie, J.B., and Balasubramaniam, S. (1985) On the effects of dietary n-3 fatty acids (maxepa) on plasma lipids and lipoproteins in patients with hyperlipidaemia. *Atherosclerosis.* **54**: 75-88.

Simons, L.A., Simons, J., McManus, P., and Dudley, J. (2000) Discontinuation rates for use of statins are high. *BMJ.* **321**: 1084-.

Simopoulos and P., A. (1999) Evolutionary aspects of omega-3 fatty acids in the food supply. *Prostaglandins Leukot. Essent. Fatty Acids.* **60**: 421-9.

Simopoulos, A.P. (1989) Summary of the nato advanced research workshop on dietary {omega}3 and {omega}6 fatty acids: Biological effects and nutritional essentiality. *J. Nutr.* **119**: 521-28.

Simopoulos, A.P. (1991) Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.* **54**: 438-63.

Skuladottir, G.V., Shi-Hua, D., Brodie, A.E., Reed, D.J., and Wander, R.C. (1994) Effects of dietary oils and methyl ethyl ketone peroxide on in vivo lipid peroxidation and antioxidants in rat heart and liver. *Lipids.* **29**: 351-7.

Smani, T., Zakharov, S.I., Csutora, P., Leno, E., Trepakova, E.S., and Bolotina, V.M. (2004) A novel mechanism for the store-operated calcium influx pathway. *Nat Cell Biol.* **6**: 113-20.

Smith, G.B., Stefenelli, T., Wu, S.T., Wikman-Coffelt, J., Parmley, W.W., and Zaugg, C.E. (1996) Rapid adaptation of myocardial calcium homeostasis to short episodes of ischemia in isolated rat hearts. *Am. Heart J.* **131**: 1106-12.

Sobel, B.E., Bresnahan, G.F., Shell, W.E., and Yoder, R.D. (1972) Estimation of infarct size in man and its relation to prognosis. *Circulation.* **46**: 640-48.

Song, J.H. and Miyazawa, T. (2001) Enhanced level of n-3 fatty acid in membrane phospholipids induces lipid peroxidation in rats fed dietary docosahexaenoic acid oil. *Atherosclerosis.* **155**: 9-18.

Song, J.H., Fujimoto, K., and Miyazawa, T. (2000) Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils. *J. Nutr.* **130**: 3028-33.

Sprecher, H. (2000) Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochim. Biophys. Acta.* **1486**: 219-31.

Steenbergen, C., Murphy, E., Watts, J.A., and London, R.E. (1990) Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. *Circ. Res.* **66**: 135-46.

Steenbergen, C., Perlman, M.E., London, R.E., and Murphy, E. (1993) Mechanism of preconditioning. Ionic alterations. *Circ. Res.* **72**: 112-25.

Steg, P.G., Francois, L., Iung, B., Himbert, D., Aubry, P., Charlier, P., Benamer, H., et al. (2005) Long-term clinical outcomes after rescue angioplasty are not different from those of successful thrombolysis for acute myocardial infarction. *Eur. Heart J.* **26**: 1831-37.

Stowe, D.F. and Kevin, L.G. (2004) Cardiac preconditioning by volatile anesthetic agents: A defining role for altered mitochondrial bioenergetics. *Antioxid Redox Signal.* **6**: 439-48.

Studer, M., Briel, M., Leimenstoll, B., Glass, T.R., and Bucher, H.C. (2005) Effect of different antilipidemic agents and diets on mortality: A systematic review. *Arch. Intern. Med.* **165**: 725-30.

Sun, J.-Z., Tang, X.-L., Park, S.-W., Qiu, Y., Turrens, J.F., and Bolli, R. (1996) Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J. Clin. Invest.* **97**: 562-76.

Supari, F., Ungerer, T., Harrison, D.G., and Williams, J.K. (1995) Fish oil treatment decreases superoxide anions in the myocardium and coronary arteries of atherosclerotic monkeys. *Circulation.* **91**: 1123-28.

Sutherland, F.J. and Hearse, D.J. (2000) The isolated blood and perfusion fluid perfused heart. *Pharmacol. Res.* **41**: 613-27.

Sutherland, F.J., Shattock, M.J., Baker, K.E., and Hearse, D.J. (2003) Mouse isolated perfused heart: Characteristics and cautions. *Clin. Exp. Pharmacol. Physiol.* **30**: 867-78.

Suzukawa, M., Abbey, M., Howe, P.R., and Nestel, P.J. (1995) Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. *J. Lipid Res.* **36**: 473-84.

Swanson, J.E. and Kinsella, J.E. (1986) Dietary n-3 polyunsaturated fatty acids: Modification of rat cardiac lipids and fatty acid composition. *J. Nutr.* **116**: 514-23.

Szekeres, L. (2005) Drug-induced delayed cardiac protection against the effects of myocardial ischemia. *Pharmacol. Ther.* **108**: 269-80.

Takahashi, R., Okumura, K., Asai, T., Hirai, T., Murakami, H., Murakami, R., Numaguchi, Y., et al. (2005) Dietary fish oil attenuates cardiac hypertrophy in lipotoxic cardiomyopathy due to systemic carnitine deficiency. *Cardiovasc Res.* **68**: 213-23.

Tamura, M., Oshino, N., Chance, B., and Silver, I.A. (1978) Optical measurements of intracellular oxygen concentration of rat heart in vitro. *Arch. Biochem. Biophys.* **191**: 8-22.

Tani, M. and Neely, J.R. (1989) Role of intracellular Na^+ in Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of H^+-Na^+ and Na^+-Ca^{2+} exchange. *Circ. Res.* **65**: 1045-56.

Tani, M., Asakura, Y., Hasegawa, H., Shinmura, K., Ebihara, Y., and Nakamura, Y. (1996) Effect of preconditioning on ryanodine-sensitive Ca^{2+} release from sarcoplasmic reticulum of rat heart. *Am J Physiol Heart Circ Physiol.* **271**: H876-81.

Tanoue, Y., Herijgers, P., Meuris, B., Verbeken, E., Leunens, V., Lox, M., and Flameng, W. (2002) Ischemic preconditioning reduces unloaded myocardial oxygen consumption in an in-vivo sheep model. *Cardiovasc Res.* **55**: 633-41.

Tavazzi, L., Maggioni, A.P., Marchioli, R., Barlera, S., Franzosi, M.G., Latini, R., Lucci, D., et al. (2008) Effect of n-3 polyunsaturated fatty acids in patients

with chronic heart failure (the GISSI-hf trial): A randomised, double-blind, placebo-controlled trial. *Lancet.* **372**: 1223-30.

Tennant, R. (1935) Factors concerned in the arrest of contraction in an ischemic myocardial area. *Am J Physiol -- Legacy Content.* **113**: 677-82.

Thannickal, V.J. and Fanburg, B.L. (2000) Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol.* **279**: L1005-28.

The, G.I. (1993) An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. *N. Engl. J. Med.* **329**: 673-82.

Thies, F., Garry, J.M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., et al. (2003) Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: A randomised controlled trial. *Lancet.* **361**: 477-85.

Thomas, M., Bashore, Christopher, B., Granger, Manesh, R., and Patel (2008), *Current medical diagnosis & treatment*, eds Stephen J, M., Maxine A, P., and Lawrence M, T. (Forty-Seventh e edn.: The McGraw-Hill Companies,). http://www.accessmedicine.com/content.aspx?aID=3671.

Tian, R. and Ingwall, J.S. (1996) Energetic basis for reduced contractile reserve in isolated rat hearts. *Am J Physiol Heart Circ Physiol.* **270**: H1207-16.

Tiidus, P.M. (1998) Radical species in inflammation and overtraining. *Can. J. Physiol. Pharmacol.* **76**: 533-8.

Tofler, G.H., Brezinski, D., Schafer, A.I., Czeisler, C.A., Rutherford, J.D., Willich, S.N., Gleason, R.E., et al. (1987) Concurrent morning increase in platelet aggregability and the risk of myocardial infarction and sudden cardiac death. *N. Engl. J. Med.* **316**: 1514-18.

Tokube, K., Kiyosue, T., and Arita, M. (1996) Openings of cardiac KATP channel by oxygen free radicals produced by xanthine oxidase reaction. *Am. J. Physiol.* **271**: H478-89.

Tomai, F., Crea, F., Chiariello, L., and Gioffre, P.A. (1999) Ischemic preconditioning in humans: Models, mediators, and clinical relevance. *Circulation.* **100**: 559-63.

Tomlinson, B. (1996) Optimal dosage of ACE inhibitors in older patients. *Drugs Aging.* **9**: 262-73.

Tosaki, A., Cordis, G.A., Szerdahelyi, P., Engelman, R.M., and Das, D.K. (1994) Effects of preconditioning on reperfusion arrhythmias, myocardial functions, formation of free radicals, and ion shifts in isolated ischemic/reperfused rat hearts. *J. Cardiovasc. Pharmacol.* **23**: 365-73.

Tritto, I., D'Andrea, D., Eramo, N., Scognamiglio, A., De Simone, C., Violante, A., Esposito, A., et al. (1997) Oxygen radicals can induce preconditioning in rabbit hearts. *Circ. Res.* **80**: 743-8.

Tsuchida, A., Thompson, R., Olsson, R.A., and Downey, J.M. (1994) The anti-infarct effect of an adenosine a1-selective agonist is diminished after

prolonged infusion as is the cardioprotective effect of ischaemic preconditioning in rabbit heart. *J. Mol. Cell. Cardiol.* **26**: 303-11.

Tuna oil [Alphabetical listing of GRAS Notices]. U.S. FDA Center for Food safety and Applied Nutrition, Office of Food Additive Safety; c2007[cited 2009 September]. Available from: http://www.cfsan.fda.gov/~rdb/opa-g109.html

Urabe, K., Miura, T., Iwamoto, T., Ogawa, T., Goto, M., Sakamoto, J., and Iimura, O. (1993) Preconditioning enhances myocardial resistance to postischaemic myocardial stunning via adenosine receptor activation. *Cardiovasc Res.* **27**: 657-62.

USDA national Nutrient Database for Standard Reference. Release 20.[homepageon the internet]. USDA, Agricultural Reaserch Service;c2007 [cited 2009 September]. Available from: http:// www.ars.usda.gov/ba/bhnrc/ndl

Valagussa, F., Franzosi, M., and Geraci, E. (1999) Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: Results of the GISSI-prevenzione trial. Gruppo italiano per lo studio della sopravvivenza nell'infarto miocardico. *Lancet.* **354**: 447-55.

Van Eyk, J.E., Powers, F., Law, W., Larue, C., Hodges, R.S., and Solaro, R.J. (1998) Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: Identification of degradation products and effects on the pCa-force relation. *Circ. Res.* **82**: 261-71.

van Kuijk, F.J., Siakotos, A.N., Fong, L.G., Stephens, R.J., and Thomas, D.W. (1995) Quantitative measurement of 4-hydroxyalkenals in oxidized low-density lipoprotein by gas chromatography-mass spectrometry. *Anal. Biochem.* **224**: 420-4.

Vanden Hoek, T., Becker, L.B., Shao, Z.H., Li, C.Q., and Schumacker, P.T. (2000) Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion. *Circ. Res.* **86**: 541-8.

Vanden Hoek, T.L., Becker, L.B., Shao, Z., Li, C., and Schumacker, P.T. (1998) Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J. Biol. Chem.* **273**: 18092-8.

Vane, J.R., Anggard, E.E., and Botting, R.M. (1990) Regulatory functions of the vascular endothelium. *N. Engl. J. Med.* **323**: 27-36.

Vanwinkle, D.M., Thornton, J.D., Downey, D.M., and Downey, J.M. (1991) The natural-history of preconditioning - cardioprotection depends on duration of transient ischemia and time to subsequent ischemia. *Coron. Artery Dis.* **2**: 613-19.

Vegh, A., Szekeres, L., and Parratt, J.R. (1990) Protective effects of preconditioning of the ischaemic myocardium involve cyclo-oxygenase products. *Cardiovasc Res.* **24**: 1020-23.

Vegh, A., Szekeres, L., and Parratt, J.R. (1991) Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc Res.* **25**: 1051-53.

Vegh, A., Szekeres, L., and Parratt, J. (1992) Preconditioning of the ischaemic myocardium; involvement of the l-arginine nitric oxide pathway. *Br. J. Pharmacol.* **107**: 648-52.

Vegh, A., Papp, J.G., and Parratt, J.R. (1994) Prevention by dexamethasone of the marked antiarrhythmic effects of preconditioning induced 20h after rapid cardiac pacing. *Br. J. Pharmacol.* **113**: 1081-2.

Venkatraman, J.T., Chandrasekar, B., Kim, J.D., and Fernandes, G. (1994) Effects of n-3 and n-6 fatty acids on the activities and expression of hepatic antioxidant enzymes in autoimmune-prone nzbxnzw f1 mice. *Lipids.* **29**: 561-8.

Verkerk, A.O., van Ginneken, A.C.G., Berecki, G., den Ruijter, H.M., Schumacher, C.A., Veldkamp, M.W., Baartscheer, A., et al. (2006) Incorporated sarcolemmal fish oil fatty acids shorten pig ventricular action potentials. *Cardiovasc Res.* **70**: 509-20.

Wajchenberg, B.L. (2000) Subcutaneous and visceral adipose tissue: Their relation to the metabolic syndrome. *Endocr. Rev.* **21**: 697-738.

Walker, D.M., Walker, J.M., Pugsley, W.B., Pattison, C.W., and Yellon, D.M. (1995) Preconditioning in isolated superfused human muscle. *J. Mol. Cell. Cardiol.* **27**: 1349-57.

Walker, D.M., Pasini, E., Kucukoglu, S., Marber, M.S., Iliodromitis, E., Ferrari, R., and Yellon, D.M. (1993) Heat stress limits infarct size in the isolated perfused rabbit heart. *Cardiovasc Res.* **27**: 962-67.

Wang, C., Harris, W.S., Chung, M., Lichtenstein, A.H., Balk, E.M., Kupelnick, B., Jordan, H.S., et al. (2006) n-3 fatty acids from fish or fish-oil supplements, but not {alpha}-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: A systematic review. *Am. J. Clin. Nutr.* **84**: 5-17.

Wang, Y., Huang, S., Sah, V.P., Ross Jr, J., Brown, J.H., Han, J., and Chien, K.R. (1998) Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J. Biol. Chem.* **273**: 2161-68.

Wang, Y.G., Benedict, W.J., Huser, J., Samarel, A.M., Blatter, L.A., and Lipsius, S.L. (2001) Brief rapid pacing depresses contractile function via Ca^{2+}/PKC-dependent signaling in cat ventricular myocytes. *Am J Physiol Heart Circ Physiol.* **280**: H90-98.

Wannamethee, G., Whincup, P.H., Shaper, A.G., Walker, M., and MacFarlane, P.W. (1995) Factors determining case fatality in myocardial infarction "Who dies in a heart attack"? *Heart.* **74**: 324-31.

Weber, C., Erl, W., Pietsch, A., Danesch, U., and Weber, P.C. (1995) Docosahexaenoic acid selectively attenuates induction of vascular cell adhesion molecule–1 and subsequent monocytic cell adhesion to human endothelial cells stimulated by tumor necrosis factor–{alpha}. *Arterioscler. Thromb. Vasc. Biol.* **15**: 622-28.

Werns, S.W. and Lucchesi, B.R. (1990) Free radicals and ischemic tissue injury. *Trends Pharmacol. Sci.* **11**: 161-6.

Weselcouch, E.O., Baird, A.J., Sleph, P.G., Dzwonczyk, S., Murray, H.N., and Grover, G.J. (1995) Endogenous catecholamines are not necessary for ischaemic preconditioning in the isolated perfused rat heart. *Cardiovasc Res.* **29**: 126-32.

West, J. (1990), *Cardiac energetics and myocardial oxygen cunsumption.In: Best and taylors physiological basis of medical practice* (12 edn.; Baltimore: Williams and Wilkins) 250-60.

Whitaker, M.O., Wyche, A., Fitzpatrick, F., Sprecher, H., and Needleman, P. (1979) Triene prostaglandins: Prostaglandin d3 and icosapentaenoic acid as potential antithrombotic substances. *PNAS.* **76**: 5919-23.

Wilber, D.J., Garan, H., Finkelstein, D., Kelly, E., Newell, J., McGovern, B., and Ruskin, J.N. (1988) Out-of-hospital cardiac arrest. Use of electrophysiologic testing in the prediction of long-term outcome. *N. Engl. J. Med.* **318**: 19-24.

Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M., and Berge, R.K. (1993) Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats. *J. Lipid Res.* **34**: 13-22.

Wissner, S.B. (1974) The effect of excess lactate upon the excitability of the sheep purkinje fiber. *J. Electrocardiol.* **7**: 17-26.

Woodcock, E.A., Lambert, K.A., and Du, X.J. (1996) Ins(1,4,5)p3 during myocardial ischemia and its relationship to the development of arrhythmias. *J. Mol. Cell. Cardiol.* **28**: 2129-38.

Xiao, Y.-F., Sigg, D.C., Ujhelyi, M.R., Wilhelm, J.J., Richardson, E.S., and Iaizzo, P.A. (2008) Pericardial delivery of omega-3 fatty acid: A novel approach to reducing myocardial infarct sizes and arrhythmias. *Am J Physiol Heart Circ Physiol.*ajpheart.91502.2007.

Xiao, Y., Kang, J.X., Morgan, J.P., and Leaf, A. (1995) Blocking effects of polyunsaturated fatty acids on Na^+ channels of neonatal rat ventricular myocytes. *PNAS.* **92**: 11000-04.

Xu, K.Y., Zweier, J.L., and Becker, L.C. (1997) Hydroxyl radical inhibits sarcoplasmic reticulum Ca^{2+}-ATPase function by direct attack on the ATP binding site. *Circ. Res.* **80**: 76-81.

Yamashita, N., Hoshida, S., Taniguchi, N., Kuzuya, T., and Hori, M. (1998) A "Second window of protection" Occurs 24 h after ischemic preconditioning in the rat heart. *J. Mol. Cell. Cardiol.* **30**: 1181-9.

Yamashita, N., Nishida, M., Hoshida, S., Kuzuya, T., Hori, M., Taniguchi, N., Kamada, T., et al. (1994) Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning. *J. Clin. Invest.* **94**: 2193-9.

Yamazaki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., et al. (1993) Mechanical loading activates mitogen-activated protein kinase and s6 peptide kinase in cultured rat cardiac myocytes. *J. Biol. Chem.* **268**: 12069-76.

Yang, B., Saldeen, T.G.P., Nichols, W.W., and Mehta, J.L. (1993) Dietary fish oil supplementation attenuates myocardial dysfunction and injury caused by global ischemia and reperfusion in isolated rat hearts. *J. Nutr.* **123**: 2067-74.

Yang, C.S. and Lee, M.J. (1987) Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies *J. Nutr. Growth Cancer.* **4**: 19-27.

Yaniv, Y., Stanley, W.C., Saidel, G.M., Cabrera, M.E., and Landesberg, A. (2008) The role of Ca^{2+} in coupling cardiac metabolism with regulation of contraction: In silico modeling. *Ann. N. Y. Acad. Sci.* **1123**: 69-78.

Yellon, D.M. and Latchman, D.S. (1992) Stress proteins and myocardial protection. *J. Mol. Cell. Cardiol.* **24**: 113-24.

Yellon, D.M. and Dana, A. (2000) The preconditioning phenomenon: A tool for the scientist or a clinical reality? *Circ. Res.* **87**: 543-50.

Yellon, D.M. and Downey, J.M. (2003) Preconditioning the myocardium: From cellular physiology to clinical cardiology. *Physiol. Rev.* **83**: 1113-51.

Yellon, D.M., Baxter, G.F., Garcia-Dorado, D., Heusch, G., and Sumeray, M.S. (1998) Ischaemic preconditioning: Present position and future directions. *Cardiovasc Res.* **37**: 21-33.

Ytrehus, K. (2000) The ischemic heart--experimental models. *Pharmacol. Res.* **42**: 193-203.

Yue, Y., Krenz, M., Cohen, M.V., Downey, J.M., and Critz, S.D. (2001) Menadione mimics the infarct-limiting effect of preconditioning in isolated rat hearts. *Am J Physiol Heart Circ Physiol.* **281**: H590-H95.

Yusuf, S., Hawken, S., Ôunpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., et al. (2004) Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the interheart study): Case-control study. *The Lancet.* **364**: 937-52.

Zaloga, G.P., Ruzmetov, N., Harvey, K.A., Terry, C., Patel, N., Stillwell, W., and Siddiqui, R. (2006) (n-3) long-chain polyunsaturated fatty acids prolong survival following myocardial infarction in rats. *J. Nutr.* **136**: 1874-78.

Zhang, J., Sasaki, S., Amano, K., and Kesteloot, H. (1999) Fish consumption and mortality from all causes, ischemic heart disease, and stroke: An ecological study. *Prev. Med.* **28**: 520-9.

Zhang, R., Khoo, M.S., Wu, Y., Yang, Y., Grueter, C.E., Ni, G., Price, E.E., et al. (2005) Calmodulin kinase ii inhibition protects against structural heart disease. *Nat. Med.* **11**: 409-17.

Zhou, X., Zhai, X., and Ashraf, M. (1996) Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window of protection by endogenous antioxidant enzyme in myocytes. *Circulation.* **93**: 1177-84.

Zhu, B.Q., Sievers, R.E., Sun, Y.P., Morse-Fisher, N., Parmley, W.W., and Wolfe, C.L. (1994) Is the reduction of myocardial infarct size by dietary fish oil the result of altered platelet function? *Am. Heart J.* **127**: 744-55.

Zimmermann, R., Andres, J., Brand, T., Frass, O., Kluge, A., Knoll, R., Vogt, A., et al. (1995) Cardiac gene expression following short coronary occlusions. *Z. Kardiol.* **84**: 159-65.

Zucchi, R., Ronca-Testoni, S., Yu, G., Galbani, P., Ronca, G., and Mariani, M. (1995) Postischemic changes in cardiac sarcoplasmic reticulum Ca^{2+} channels : A possible mechanism of ischemic preconditioning. *Circ. Res.* **76**: 1049-56.

APPENDIX

Appendix 3.1 Krebs Henseleit Solution

1. Weigh out the following amount of each chemical either as 1L or 2L concentrated (10×) stock ($CaCI_2$) is added on day of use: see below).

		Final solution		**10×Concentration**	**10×Concentration**
	Mwt	**mM**	**g/litre**	**1litre**	**2 litre**
NaCI	58.44	118.0	6.92	69.2	138.4
KCI	74.55	4.7	0.35	3.5	7.0
$MgSO_4.7H_2O$	246.47	1.64	0.29	2.9	5.8
KH_2PO_4	136.09	1.18	0.16	1.6	3.2
$NaHCO_3$	84.01	24.88	2.10	21.0	42.0
Glucose	180.16	11.10	2.00	20.0	40.0

2. Dissolve each salt in Milli-Q water completely before the addition of the next salt.

3. Make up to the required volume in a volumetric flask.

4. Store in the fridge in a clean Winchester or similar container[will keep as a concentrated stock solution for about 2 weeks](can be kept longer if glucose is omitted from stock and added only at time of use).

5. Dilute 10×concentration with Milli-Q water (e.g. dilute 200mL to almost 2L in volumetric flask).

6. Bubble with carbogen gas (5%CO2, 95%O2) for 30 minutes.

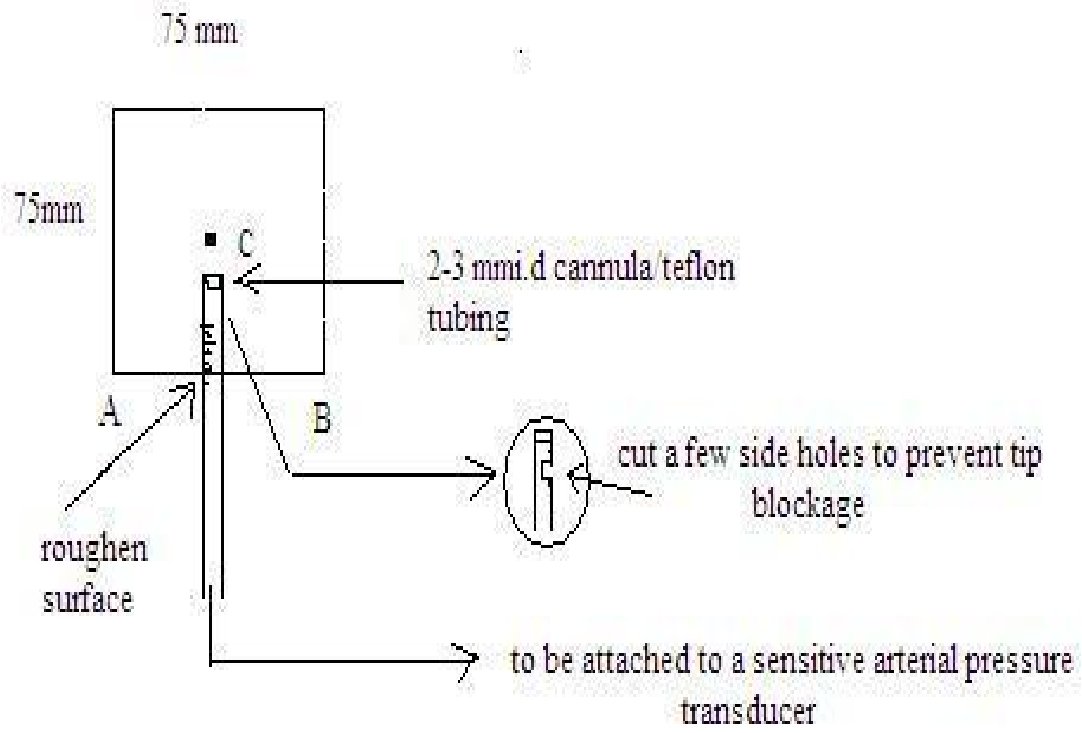
7. Add 1.5mL $CaCI_2$ per liter(final concentration 1.5mM).

8. Make up to final volume (e.g. 2L)

9. Solution is ready, filter it before use.

Appendix 3.2 Schematic diagram of making balloon from biofilm

Step one. Cut a 75mm*75mm cling wrap and place a transducer 2-5 mm away from centre of the cling wrap (shown in the diagram below).

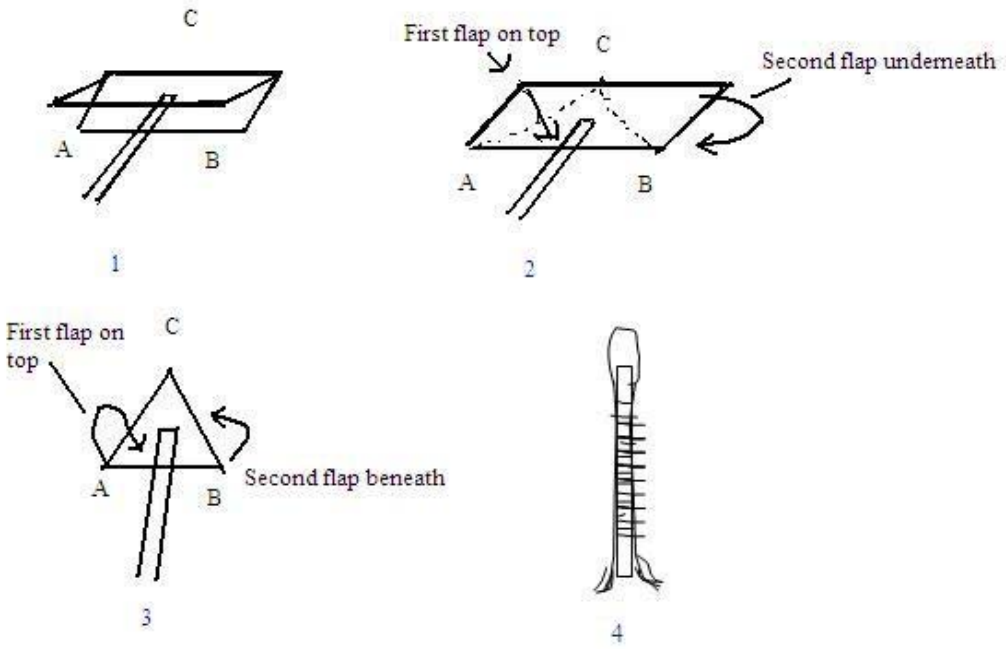


* Cannula must remain precise as positioned between A and B throughout folding.

* Tip of the cannula must be kept a minimum of 2-5mm away from centre.

Step two. Fold cling wrap according to the diagram below

Figure1.6
Proposed Mechanism



Eventually end up with smaller and smaller triangular flaps that have been folded alternately (top/bottom), keep folding until reach the point where can not be fold anymore.

Step 3. Choose a long silk suture and wrap the folded cling wrap with the transducer, wrap as close as possible, no space should be visible between each thread. Trim excess plastic if too much is still out.

*: Distance between last wrap of thread and tip of "balloon" should be about 5mm but ultimately depends on heart size.