Abstract

Coronary artery disease is the leading cause of death in North America. The invasiveness of its treatment depends on its severity; less severe disease can be treated pharmacologically or surgically without significantly different outcomes, but coronary artery bypass grafting (CABG) clearly reduces mortality among medium- and high-risk patients compared to percutaneous and non-surgical intervention.

Although the majority of patients undergoing surgical revascularization emerge without severe postoperative complications, a significant portion of patients encounter a postoperative complication known as low cardiac output syndrome which can quadruple the overall mortality rate for CABG. Intraoperative ischemia reperfusion injury is a major factor in the development of low cardiac output syndrome; so effective intraoperative myocardial protection is central to reducing its incidence, and represents an opportunity to considerably improve patient outcomes.

The introductory chapter of this thesis describes the origin and role of reactive oxygen species (ROS) in myocardial ischemia-reperfusion injury. In addition, it introduces key strategies targeted to reduce ROS-mediated myocardial ischemia-reperfusion injury, highlighting key clinical studies that translated these strategies to reduce the severity of ischemia-reperfusion injury during CABG.

The central hypothesis of the clinical project on which this thesis is based states that propofol reduces the incidence of low cardiac output syndrome subsequent to CABG with CPB by decreasing the magnitude of 15-F$_{2\alpha}$-isoprostane generation during ischemia-reperfusion. The second chapter introduces propofol, and will review previous studies that explore its cardioprotective potential.
The experimental section of this thesis describes the development of a quantitative technique for propofol analysis in whole blood, and its application in a dose finding study that define the parameters for achieving experimentally relevant concentrations of propofol during cardiopulmonary bypass. These two studies were fundamental to the development of a clinical study evaluating ROS generation and the incidence low cardiac output syndrome in patients undergoing CABG surgery.

Preliminary results that address the central hypothesis are subsequently presented, along with an alternative proposed mechanism for propofol-mediated cardioprotection. This thesis will conclude with a summary of findings and a description of several future studies aimed at testing, generating, and evaluating new hypotheses.
Preface

The author, Koen Raedschelders, performed the majority of the research and analysis leading to the results included in this dissertation, and performed the entirety of the writing. The contributions of other researchers and collaborations are summarized below:

Contributions from other researchers:

Chapter 3: Yu Hui and Koen Raedschelders performed linearity, reproducibility, and precision analyses, and analyzed patient whole blood samples. Hong Zhang modified the liquid-liquid extraction to make it amenable for capillary electrophoresis separations and performed exploratory separations.

Chapter 4: Yu Hui and Hong Zhang analyzed patient whole blood samples. Bradley Laferlita was the anesthesiology resident responsible for hemodynamic monitoring while Tao Luo was responsible for the study protocol.

Chapter 5: David Ansley, the principal investigator, developed the study described in this chapter with input from Peter Choi.

Chapter 6: Yu Hui performed quantitative propofol analyses, 15-F2\textsubscript{2}isoprostane analyses, and 3-nitrotyrosine assays. NO\textsubscript{2}\textsuperscript{-} assays were performed in the laboratory of Pascal Bernatchez.

Ethics approval:

This study was reviewed by and received approval from the UBC Clinical Research Ethics Board (Certificate number H04-70456)
Publications arising from work presented in the dissertation:


   Material from this article is included in Chapter 3


   Material from this article is included in Chapter 4


   Material from this article is included in Chapter 5
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BH₂</td>
<td>Dihydrobiopterin</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CK-MB</td>
<td>Myocardial creatine kinase</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CPB</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FAD/FADH₂</td>
<td>Flavin adenine dinucleotide: oxidized / reduced</td>
</tr>
<tr>
<td>FMN/FMNH⁺</td>
<td>Flavin mononucleotide oxidized / reduced semiquinone radical</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HbO₂</td>
<td>Oxygenated Hemoglobin</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>L₀</td>
<td>Capillary length from inlet to the detection window, in CE</td>
</tr>
<tr>
<td>Lₜ</td>
<td>Total capillary length from, in CE</td>
</tr>
<tr>
<td>L-NAME</td>
<td>$N^G$-nitro-Levo-arginine methyl ester; competitive NOS inhibitor</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>$N^G$-monomethyl-Levo-arginine; competitive NOS inhibitor</td>
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<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar electrokinetic capillary chromatography</td>
</tr>
<tr>
<td>MetHb</td>
<td>Hemoglobin with ferric (as opposed to ferrous) iron</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>NAD(P)+/NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide (phosphate): oxidized / reduced</td>
</tr>
<tr>
<td>NO’</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>Nitrogen dioxide radical</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>ONOOOH</td>
<td>Peroxynitrous acid</td>
</tr>
<tr>
<td>O₂</td>
<td>Molecular Oxygen</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>prn</td>
<td>Denotes “as per routine practice”</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species: Encompasses O₂⁻, NO’, NO₂⁻⁻, ONOO-, and ONOOOH</td>
</tr>
<tr>
<td>Rᵈ</td>
<td>Resolution</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>tᵅmig</td>
<td>Migration time (of a given analyte)</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>UQ/ UQH⁺</td>
<td>Ubiquinone: oxidized quinone / reduced semiquinone</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
<tr>
<td>σ</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>μ</td>
<td>Mean</td>
</tr>
<tr>
<td>μₑᵖᴬ</td>
<td>Electrophoretic mobility (in this case, of analyte “A”)</td>
</tr>
<tr>
<td>Ψₘ</td>
<td>Inner mitochondrial membrane potential</td>
</tr>
</tbody>
</table>
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“Every child knows that play is nobler than work.”

–Cormac McCarthy. Blood Meridian

This thesis has no dedication. That nobler pursuit is most often dedicated to Monique Raedschelders -proud, principled, and independent. A pure fire that grew until her body could no longer contain the flames, and her wild soul was set free.
1. **Introduction to reactive oxygen species generation during myocardial ischemia and reperfusion**

Coronary artery disease is the leading cause of death in North America\textsuperscript{1,2}, accounting for an estimated direct and indirect cost of over $448.5 billion\textsuperscript{2}, and $18.5 billion in Canada\textsuperscript{3}. Less severe coronary artery disease can be treated pharmacologically or surgically without significant differences in outcomes\textsuperscript{4,5}, but coronary artery bypass grafting (CABG) clearly reduces mortality among medium- and high-risk patients, including diabetics\textsuperscript{6,7}, over non-surgical management\textsuperscript{8} and percutaneous interventions\textsuperscript{6,9,10}. The 515,000 annual CABG surgeries done in the United States alone testify to the effectiveness of this technique\textsuperscript{11}.

Although the majority of patients undergoing surgical revascularization emerge without severe postoperative complications, a significant proportion of patients encounter a postoperative complication known as low cardiac output syndrome. Low cardiac output syndrome can quadruple the overall mortality rate for CABG from 2\% to 8\%\textsuperscript{12,13}. Intraoperative ischemia-reperfusion injury is a major factor in the development of low cardiac output syndrome; so effective intraoperative myocardial protection is central to reducing the incidence of this high-risk scenario. Low cardiac output syndrome affects up to 26\% of diabetic patients, compared to 8\% to 15\% of non-diabetic patients recovering from cardiac surgery\textsuperscript{14-16}. Several factors likely contribute to this discrepancy, chief among these are defective antioxidant defenses\textsuperscript{17,18}, increased oxidative stress, and impaired endogenous myocardial protective pathways identified in the diabetic heart\textsuperscript{19,20}. Diabetics are two to five times more likely to develop cardiovascular disease and therefore account for up to 30\% of open heart procedures\textsuperscript{21}. Diabetic patients are
particularly at an elevated risk of complications after CABG and accordingly have a lower 10-year survival\(^\text{16}\). Managing ischemia-reperfusion injury during to CABG represents an opportunity to considerably improve patient outcomes.

1.1. **Myocardial ischemia-reperfusion injury**

During CABG with cardiopulmonary bypass (CPB), venous blood is redirected from the vena cava to the “heart-lung machine”, which mediates artificial gas exchange and pumps oxygenated blood back through the body via the aorta. The heart-lung machine provides the body with adequate perfusion through constant delivery of oxygenated blood downstream of the aorta, but cardiac perfusion is a direct physical consequence of cardiac systole/diastole cycling. Cardioplegia solution is administered through the coronary vasculature to electrically silence the heart during CPB, thus inhibiting excitation-contraction and reducing myocardial energy demand. Despite the administration of cardioplegia and the absence of contraction, the myocardium still consumes oxygen at a rate of 2 mL-of-O\(_2\)-per-100g-per-min\(^\text{22}\). When the heart is electrically silenced, its oxygen and nutrient demands are decreased, but its blood flow is also effectively halted. The resulting ischemia can range in severity from reversible to fatal, and as the duration and severity of ischemia increases, so does the extent of myocyte injury and death\(^\text{23, 24}\).

Ischemic injury is more complex than O\(_2\) and ATP depletion, and CO\(_2\) buildup; these events simply represent the first consequences of ischemia, which in turn induce a myriad of changes at the cellular, tissue, and organ levels. Over 95% of ATP production in the non-ischemic heart results from mitochondrial oxidative phosphorylation, the vast majority being fuelled by fatty acid β-oxidation\(^\text{25}\). The insufficient blood supply during ischemia causes oxidative substrates to accumulate at the expense of their reductive
counterparts; the myocardial NADH/NAD+ concentration ratio can increase more than 10-fold during ischemia.²⁶

In response, the heart shifts its metabolic phenotype from β-oxidation towards glycolysis. This phenotypic shift is accomplished in part by increasing the expression of the GLUT-1 and GLUT-4 transporters²⁷-²⁹ and increasing glycogenolysis³⁰, and is stimulated by the increase in inorganic phosphates and decreased pH that accompany ischemia. The final metabolite of glycolysis is pyruvate, but at low oxygen tension, pyruvate is used as an electron acceptor and reduced to lactate. Non-mitochondrial ATP turnover during ischemic episodes, combined with the accumulation of lactate from glycolysis at low oxygen tension, results in ischemic intracellular acidosis.³¹

Glycolysis is a short-term solution to the problem of ischemia because myocardial glucose stores are limited.³² Aside from changing their metabolic phenotype, ischemic myocytes can re-establish their oxygen demand to meet its decreased supply by reducing contractility with a generalized downgrading of cardiac function. This process results in myocardial hibernation.³³ Even in the less active hibernating state, myocytes still require ATP for housekeeping processes like ion homeostasis. If oxygen supplies are depleted below the levels required for hibernation, then the myocardium will sustain prolonged dysfunction.

Ion homeostasis is the major metabolic priority for the heart. This remains true during myocardial ischemia, hibernation, and stunning alike. The myocardium requires ATP to restore the limited energy reserves that are quickly exhausted during ischemia. Ion homeostasis depends primarily on key energy-dependent ion exchangers including the Na⁺/K⁺ ATPase, and Ca²⁺-ATPases. Secondary channels include the Na⁺/Ca²⁺ and the
Na\textsuperscript{+}/H\textsuperscript{+} exchanger. Proton accumulation resulting from unbalanced ATP hydrolysis drives the Na\textsuperscript{+}/H\textsuperscript{+} exchanger leading to an initial intracellular Na\textsuperscript{+} overload, and an eventual intracellular Ca\textsuperscript{2+} overload (Figure 1) (Reviewed in\textsuperscript{34,35}). Myocardial acidosis reduces cardiac contractility by interfering with the normal Ca\textsuperscript{2+}-troponin C interaction\textsuperscript{36}, thus promoting arrhythmia. Ultimately, ischemia results in energy starved, Ca\textsuperscript{2+}-loaded, acidic myocytes. These conditions strongly contribute to ischemic arrhythmia\textsuperscript{37}, contraction failure\textsuperscript{38}, and cell death\textsuperscript{39}.
Figure 1: i) Myocardial ionic alterations during myocardial ischemia. 1. Depolarization via $\text{Na}^+$ influx; 2. $\text{Ca}^{2+}$ influx/excitation-contraction coupling via (a) voltage gated $\text{Ca}^{2+}$ influx and (b) Ryanodine receptor mediated $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release; 3. Repolarization via $\text{K}^+$ efflux; 4. Re-establishing electrochemical gradients via (a) $\text{Na}^+/\text{Ca}^{2+}$ exchange, (b) ATP-driven $\text{Ca}^{2+}$ sequestration, (c) ATP-driven $\text{Na}^+/\text{K}^+$ exchange; 5. Acidosis-induced $\text{Na}^+/\text{H}^+$ exchange. ii) Depiction of the role that altered cellular energetic plays in promoting ischemic acidosis and altered myocardial ion fluxes.
The principle that timely reperfusion could salvage the ischemic myocardium, and that the extent of ischemia was reflected by the extent of the myocardial apoptosis and necrosis was clearly defined in the mid 1970’s. Additionally, the role of calcium overload and contracture had been established by the finding that verapamil and propranolol could influence post-ischemic myocardial cell death. Nevertheless, these results contrasted with a 1973 report describing a massive protein and enzyme release from the myocardium immediately after aerobic reperfusion. The effect of reperfusion was subsequently characterized by ionic and histological differences that were not present during ischemia. Namely, post-ischemic reperfused myocytes showed increased intracellular Na⁺, Cl⁻, and Ca²⁺ concentrations, as well as decreased intracellular K⁺ concentrations, with large contractile proteins forming distinct bands while mitochondria (and indeed cells) became swollen. Several studies in the late 1970’s and early 1980’s further described reperfusion injury from a functional perspective in animal models, and even from the perspective of CABG. Despite these observations, the concept that a distinct injury was generated at reperfusion initially remained controversial. A major obstacle was that reperfusion injury, at that time, was a concept that lacked a mechanism.

1.2. Free radical generation during myocardial ischemia-reperfusion

The concept that the reintroduction of molecular oxygen to the myocardium could induce a unique type of injury was first proposed by Hearse et al in 1973 who noticed that a large fraction of cellular enzymes were released not during hypoxia, but rather upon sudden reoxygenation. The concept was further supported when the inclusion of exogenous glucose to anoxic perfusate to supplement the limited endogenous myocardial
supply was found to relieve cell injury at reperfusion\textsuperscript{50, 51}. Thus, myocyte injury subsequent to post-ischemic reperfusion was paradoxically predicated on the reintroduction of oxygen to cells that are energy-starved, which clearly implicated cellular metabolism. In 1980, Guarnieri \textit{et al} demonstrated that ischemia and reperfusion impaired superoxide dismutase activity and decreased cellular glutathione-to-glutathione disulfide ratio (see equations 3 and 5) while increasing lipid peroxidation, suggesting that the extent of superoxide anion radical (O$_2$$^•$-) produced at reperfusion exceeded the capacity of endogenous cellular antioxidant systems\textsuperscript{52}. The overall results of these and other similar studies introduced the notion that metabolic reducing equivalents accumulate during ischemia, which in turn serve as substrates for oxygen-centered free radical generation at reperfusion\textsuperscript{53}. Experiments using isolated heart models in the presence or absence of superoxide dismutase further implicated oxygen-derived radicals as likely mediators of reperfusion injury\textsuperscript{54-56}, but due to the lack of direct evidence of production, identity, and mechanism of action, their role remained controversial.

In the late 1980’s, the application of electron spin resonance and spin trapping directly implicated oxygen-derived free radicals in ischemia-reperfusion injury\textsuperscript{57-63}. Pioneering work using electron spin resonance spectroscopy for the first time directly characterized the generation of oxygen-, nitrogen-, and carbon-centered free radicals during ischemia and reperfusion in an isolated rabbit heart model\textsuperscript{57}. Similar results were independently produced in isolated rat model\textsuperscript{59}. Although these spin trapping experiments could not definitively identify the radicals that were generated, they clearly showed that the signals for oxygen- and nitrogen-centered radicals increased marginally during ischemia at the apparent expense of the carbon-centered semiquinone radical signal\textsuperscript{57}. 


The ischemic pattern of reactive oxygen and nitrogen species (collectively ROS) generation was unchanged with hypoxic reperfusion, but all three signals spiked with oxygenated reperfusion; their peak occurring approximately 20 seconds thereafter. Arroyo et al subsequently used spin trapping to identify O$_2^\cdot$\superscript{−}, hydroxyl radical ('OH), and a carbon centered radical at reperfusion, and found that their cumulative generation at reperfusion was abolished by superoxide-dismutase. Accordingly, O$_2^\cdot$\superscript{−} was identified as the parent radical that serves as a precursor to the formation of both 'OH and the carbon-centered radical\superscript{58} which the same group subsequently identified as a group of oxy-radical-mediated lipid peroxidation products\superscript{64}. Bolli et al demonstrated that this pattern of free-radical generation extended to an intact dog model\superscript{60}, in which O$_2^\cdot$\superscript{−} was similarly identified as the parent radical at reperfusion\superscript{61}.

The advent of techniques capable of direct detection and identification of oxygen-centered radicals in the late 1980’s was critical to establish their generation during myocardial ischemia and reperfusion. Subsequent studies, in which exogenously administered ROS at levels akin to those observed during reperfusion induced similar calcium overloading, functional depression, and metabolic changes\superscript{65, 66}, cemented the ROS as central mediators of this injury.

1.3. Sources of superoxide during myocardial ischemia and reperfusion

O$_2$ consists of two oxygen atoms with an electron configuration containing two unpaired electrons in separate orbitals. As such, O$_2$ is a diradical with a high electron affinity and is itself relatively reactive. O$_2$ is completely reduced by accepting four electrons to yield two water molecules. Alternatively, partially reduced oxygen yields highly unstable intermediates. The addition of a single electron to O$_2$ yields O$_2^\cdot$\superscript{−}, the
addition of another electron in an aqueous environment yields hydrogen peroxide (H$_2$O$_2$), which can get further reduced to the highly reactive 'OH. Each of these partially reduced forms of oxygen are generated during ischemia and reperfusion, but experiments using superoxide dismutase clearly indicate that O$_2$$^-$ is the parent radical - without it, neither H$_2$O$_2$ or 'OH are appreciably generated$^{58,61}$. Low levels of O$_2$$^-$ are generated mainly by electron leakage within the mitochondrial electron transport chains of normally functioning cells. In contrast, O$_2$$^-$ generation during ischemia and reperfusion occurs on a much larger scale, with multiple potential cellular sources.
**Normoxia**

Mitochondrial electron transport chain activity

**Ischemia**

Prolonged ischemia

**Reperfusion**

(NADPH cycle)

---

**Cellular Xanthine Oxidase**

- Hypoxanthine → Xanthine dehydrogenase → Xanthine → Xanthine oxidase → Uric acid
- NADPH

**Immune system response**

**Uncoupled NOS**

- L-arginine → NOS + BH₄ → L-citrulline + NO
- NADPH + O₂

- Tetrahydrobiopterin (BH₄) → Prolonged ischemia → Dehydrobiopterin (BH₂) → NADPH
**Figure 2:** Major sources of $O_2^\cdot$ during myocardial ischemia and reperfusion. *Top panel left:* Tricarboxylic acid cycle (TCA) coupled to complete $O_2$ reduction at complex IV with electron flux ($e^\cdot$) through mitochondrial electron transport complexes. $H^+$ pumping at complex I, III, and IV enables ATP synthesis. *Top panel centre:* Ischemic inhibition of electron flux beyond complex III and decreased TCA cycle activity results in a modest increase in $O_2^\cdot$ generation primarily at complex III, decreased $H^+$ pumping, and $F_1F_0$-ATPase reversal. *Top panel right:* TCA cycle reactivation at reperfusion with sustained electron flux inhibition beyond complex III increases $O_2^\cdot$ generation at complex III. $H^+$ pumping at complex I and III weakly reestablishes the proton motive force and enables low-level ATP synthesis. *Middle panel left:* Xanthine dehydrogenase activity under normal perfusion. *Mid panel centre/right:* Prolonged ischemia converts xanthine dehydrogenase to xanthine oxidase, which mediated $O_2^\cdot$ generation. *2nd centre panel right:* NAD(P)H dehydrogenase in activated immune cells produces $O_2^\cdot$ generation subsequent to cell damage. *Lower panel left:* Coupled constitutive nitric oxide synthase (NOS) production of NO$. Lower panel centre:* Prolonged ischemia results in the oxidation of tetrahydrobiopterin (BH$_4$), a NOS cofactor, to yield dihydrobiopterin (BH$_2$). *Lower panel centre/right:* Uncoupled constitutive NOS-BH$_2$ results in $O_2^\cdot$ generation.
1.3.1 Mitochondrial electron transport

The mitochondrial electron transport chain is a series of enzyme complexes that utilize the free energy released by a succession of spontaneous redox reactions to generate a proton gradient during the inner mitochondrial membrane. The high-energy intermediates that fuel this process are NADH and FADH$_2$, which are supplied by the tricarboxylic acid cycle and ultimately lead to the reduction of molecular oxygen to water. During mitochondrial oxidative phosphorylation, the enzyme F$_1$F$_0$-ATP synthase uses the potential energy stored within the mitochondrial electrochemical proton motive force to generate ATP.

The generation of the mitochondrial proton motive force is entirely dependent on the rapid cycling of successive redox intermediates within the mitochondrial electron transport chain. The complexes of the electron transport chain undergo reduction and oxidation in order of increasing reduction potentials in a highly regulated and tightly coupled process. When the system is functioning normally, electron transport is initiated with the oxidation of NADH by the NADH dehydrogenase complex and virtually always results in the complete reduction of oxygen to water at the cytochrome oxidase complex.

Only an estimated 1-2% of electrons passing through the mitochondrial electron transport chain under normal physiological conditions result in the production of O$_2$. The consistent leakage of electrons from the electron transport chain to produce O$_2$ has fueled speculation of their role as cell signaling intermediates. To this end, Aon et al suggest that this physiological superoxide production is part of an oscillating O$_2$ pattern which serves a critical role to couple myocardial energy production with energy demand. Indeed, O$_2$ generated under
normal physiological conditions regulates important cell functions including metabolism, proliferation, and apoptosis (reviewed in). Mitochondrial O$_2^\cdot$ generation occurs in the inner mitochondrial membrane by a non-enzymatic, single electron transfer to O$_2$ primarily by ubisemiquinone (equation 1) in complex III and secondarily by reduced flavin mononucleotide in the NADH dehydrogenase complex (equation 2). Most cells contain enzymatic antioxidant defense mechanisms that quickly convert ROS to water. Specifically, superoxide dismutase enzymes, which contain either copper, manganese, or a nickel metal centers that can be either reduced or oxidized to convert O$_2^\cdot$ to O$_2$ and H$_2$O$_2$ in a reaction that is effectively diffusion-limited (equation 3). H$_2$O$_2$ is subsequently converted to water by either catalase (equation 4) or by the glutathione peroxidase system (equation 5). Variations of these endogenous antioxidant enzyme systems are expressed in both the mitochondrial matrix and the cytoplasm of their host cells in the myocardium.

Equation 1: \[ UQH^- + O_2 \rightarrow UQ + H^+ + O_2^- \]

Equation 2: \[ FMNH^- + O_2 \rightarrow FMN + H^+ + O_2^- \]

Equation 3(a/b): \[ SOD^{(n+1)+} + O_2^- \rightarrow SOD^{(n)} + O_2 \]
\[ SOD^{(n)} + O_2^- + 2H^+ \rightarrow SOD^{(n+1)+} + H_2O_2 \]

Equation 4(a/b): \[ CAT(Fe^{III}) + H_2O_2 \rightarrow CAT(Fe^{IV} = O) + H_2O \]
\[ CAT(Fe^{IV} = O) + H_2O_2 \rightarrow CAT(Fe^{III}) + H_2O + O_2 \]

Equation 5(a/b): \[ H_2O_2 + 2(GSH) \xrightarrow{\text{Glut peroxidase}} GSSG + 2H_2O \]
\[ GSSG + NADPH + H^+ \xrightarrow{\text{Glut reductase}} 2(GSH) + NADP^+ \]

The idea that myocardial injury results from the ROS burst at reperfusion is somewhat misleading. Sub-lethal levels of ROS are clearly generated in the ischemic myocardium. ROS generated during ischemia induce peroxidation of cardiolipin, a
mitochondrial lipid integral to the stability and activity cytochrome oxidase dimers\textsuperscript{80, 81}. Ischemic inhibition of cytochrome oxidase prevents electron flux beyond the level of cytochrome c\textsuperscript{80, 82-84}, and causes redox intermediate cycling to cease. A new equilibrium establishes under these conditions in which upstream intermediates predominantly exist in their reduced form and in which partial $O_2$ reduction to $O_2^{-}$ by ubisemiquinones of complex III is favored. The result is a modest increase in $O_2^{-}$ generation during ischemia\textsuperscript{79}.

Complex III of the mitochondrial electron transport chain contains two ubisemiquinone moieties; $Q_0$ and $Q_i$\textsuperscript{85, 86}. Ischemic damage also occurs at complex III\textsuperscript{87}, and this damage appears to enhance $O_2^{-}$ generation at the $Q_0$ site which is oriented toward the intermembrane space where cellular and mitochondrial antioxidant defenses are not present\textsuperscript{72, 88}. By contrast, $O_2^{-}$ generated at the $Q_i$ site, like that generated by NADH dehydrogenase, is more likely to enter the mitochondrial matrix, where it would quickly be met with mitochondrial antioxidant defenses\textsuperscript{72, 86, 88}. $O_2^{-}$ released into the intermembrane space can diffuse into the cytosol in its neutral protonated form (HO$_2^-$) or through the voltage-dependent anion channel\textsuperscript{89}. The generation of $O_2^{-}$ during brief ischemia is thought to play an important role in preconditioning\textsuperscript{90-92} and may also play a role in post-reperfusion energy recovery\textsuperscript{93}, so its generation and release to the intermembrane space by $Q_0$ may be important from a signaling perspective.

Alternatively, cardiolipin peroxidation and cytochrome oxidase uncoupling originating during ischemia persists into reperfusion, at which time equation 1 becomes the \emph{de facto} terminal redox step for damaged mitochondrial electron transport. In contrast with ischemia, tricarboxylic acid cycle intermediates and $O_2$ tension are readily
replenished at reperfusion, and $\text{O}_2^-$ generated from damaged electron transport chains and subsequent $\text{H}_2\text{O}_2$ production by superoxide dismutase are free to occur on a much larger scale$^{94}$. In this way, radicals generated during ischemia work in concert with the oxidative burst at reperfusion to mediate ischemia-reperfusion injury in cardiomyocytes$^{95}$.

$\text{O}_2^-$ generation during ischemia appears to be a self-limiting phenomenon; decreased flux through the tricarboxylic acid cycle does not lend itself to efficiently supplying single electrons to ubisemiquinone after it leaks them to $\text{O}_2$. This reasoning extends to Zweier’s original electron spin resonance observation of a marginal increase in the alkyl-peroxyl centered radical signal during ischemia at the apparent expense of the carbon-centered semiquinone signal$^{57}$, and also extends to a subsequent study, in which ischemic alkyl-peroxyl and $\text{O}_2^-$ concentrations reach a plateau rather than increasing indefinitely with prolonged ischemia$^{78}$.

The energy demand of the myocytes is reflected by their large mitochondrial content$^{96}$, which makes these cells especially vulnerable to electron transport chain damage and to increased mitochondrial $\text{O}_2^-$ generation during ischemia and reperfusion$^{97,98}$. The consequences of this persistent mitochondrial damage are exemplified by the finding that cardiac work, normalized to $\text{O}_2$ consumption or tricarboxylic acid cycle activity, only fractionally recovers at reperfusion$^{99}$. If ischemia is brief, a relatively large fraction of mitochondrial electron transport will catalyze the complete reduction of oxygen at reperfusion. If ischemia is more severe, the proportion of damaged mitochondrial electron transport proteins increases along with the amount of $\text{O}_2^-$ that is produced at early reperfusion (Figure 2).
1.3.2  Cellular xanthine oxidase

The mitochondrial electron transport chain is an important source of $O_2^{\bullet-}$ during ischemia and reperfusion, but several other sources also contribute. The xanthine dehydrogenase/xanthine oxidase enzyme system was identified early as a potential source of cellular $O_2^{\bullet-}$ generation$^{100,101}$. Xanthine dehydrogenase catalyzes the catabolic oxidation of hypoxanthine to xanthine and subsequently to uric acid by coupling the reaction with NAD$^+$ reduction to yield NADH (equation 6). During ischemia, xanthine dehydrogenase can be converted to xanthine oxidase by the modification of a sulfhydryl group$^{102}$ or by proteolytic cleavage$^{103}$. Although xanthine oxidase is still capable of catalyzing the conversion of hypoxanthine to uric acid, it does so by coupling the reaction with the reduction of molecular oxygen$^{104}$ (equation 7). The cessation of oxidative phosphorylation during ischemia causes ATP degradation to successively lower energy products of purine catabolism, of which hypoxanthine is an intermediate. Thus, hypoxanthine accumulates during ischemia, and serves as a substrate for equation 7a when $O_2$ tension is restored at reperfusion. Equation 7a also provides xanthine as a substrate for equation 7b at reperfusion.

Equation 6 (a/b): \[
\text{hypoxanthine} + \text{NAD}^+ \xrightarrow{\text{XDH}} \text{xanthine} + \text{NADH} \\
\text{xanthine} + \text{NAD}^+ \xrightarrow{\text{XDH}} \text{uric acid} + \text{NADH}
\]

Equation 7 (a/b): \[
\text{hypoxanthine} + O_2 \xrightarrow{\text{XO}} \text{xanthine} + O_2^{\bullet-} \\
\text{xanthine} + O_2 \xrightarrow{\text{XO}} \text{uric acid} + O_2^{\bullet-}
\]

The contribution of xanthine oxidase as a significant source of $O_2^{\bullet-}$ generation during ischemia and reperfusion remains rather controversial. The xanthine oxidase inhibitor allopurinol was shown to preserve myocardial glutathione and catalase activity and decreased infarct size in separate dog models of ischemia-reperfusion injury in the
1980’s\textsuperscript{105, 106}. Shortly thereafter, Grum et al. failed to detect any appreciable xanthine oxidase or dehydrogenase activity in the rabbit heart\textsuperscript{107}. Similarly undetectable xanthine oxidase activity was reported in the human heart\textsuperscript{108, 109}, and its contribution to post-ischemic $\text{O}_2^•$ production in the rabbit heart was discounted\textsuperscript{110}. These results contradict allopurinol-mediated free-radical reduction. Xanthine oxidase is readily expressed in endothelial cells that line the myocardial vasculature\textsuperscript{111, 112}, thus allopurinol may protect the myocardium by reducing post-ischemic $\text{O}_2^•$ production in endothelial cells. Alternatively, allopurinol has been shown to improve post-ischemic myocardial function independently of xanthine oxidase inhibition\textsuperscript{113-115}, possibly through an intrinsic antioxidant activity\textsuperscript{116} or cellular metabolic modulation\textsuperscript{117, 118}. These secondary effects are more likely explanations for allopurinol-mediated protective effects recently reported in patients undergoing percutaneous intervention following acute myocardial infarction\textsuperscript{119}.

Xanthine dehydrogenase activity was found to be relatively stable in human heart tissue\textsuperscript{120}, and its conversion to xanthine oxidase is a relatively slow process with a half-life of 7 hours in ischemic rat tissue\textsuperscript{103}. This xanthine dehydrogenase stability suggests that xanthine oxidase is unlikely to be appreciably formed during transient ischemia. By the same token, one would expect xanthine oxidase levels to increase under prolonged pathological conditions such as ischemic heart disease (Figure 2). This is in agreement with previously reported results\textsuperscript{121}, and therefore provides mechanistic support to reports of allopurinol-mediated reductions in $\text{O}_2^•$ markers with improved functional recovery within the context of CABG\textsuperscript{122} and improved exercise tolerance in patients with chronic stable angina\textsuperscript{123}. 
The immune system

The spin-trap and resonance studies that definitively identified \( O_2^- \) as the parent free radical produced in the post-ischemic heart showed an important difference between experimental models: isolated heart models had maximal free-radical intensities within a minute of reperfusion before quickly dropping to near-baseline levels\(^{57-59, 62, 64, 78, 124}\), while similar experiments in intact animals showed that the oxidative burst was still elevated 3 hours after reperfusion\(^{60, 61}\). This discrepancy in the timeframe for ROS generation between isolated heart and whole animal models illustrated that whole blood either prolongs myocardial \( O_2^- \) generation, or contains components that, once activated by the post-ischemic myocardium, take over \( O_2^- \) production. It is in this context that the cells of the immune system are another source of post-ischemic myocardial \( O_2^- \) generation.

The activation of the immune system at reperfusion in response to myocardial injury is a stepwise process whose magnitude depends on the extent of myocyte and endothelial cell damage. Myocardial damage incurred during ischemia and early reperfusion activates the complement system\(^{125, 126}\), which in turn leads to the secretion of strong chemotactic stimuli and the expression of proteins that promote infiltration of phagocytes (reviewed in\(^{127}\)).

The contribution of phagocytes to post-ischemic myocardial \( O_2^- \) generation occurs primarily as a result of neutrophil infiltration\(^{127-129}\). These cells contain the enzyme NAD(P)H oxidase, which can use either NADH or NADPH as substrates for the single electron reduction of \( O_2 \) (equation 8, Figure 2)\(^{130}\).

Equation 8: \[
NAD(P)H + 2O_2 \xrightleftharpoons{NAD(P)H\_oxidase} NAD(P)^+ + H^+ + 2O_2^-
\]
The generation of $O_2^{-}$ by the immune system is less a consequence of ischemia and reperfusion than an immune response to tissue and cell damage. As such, one would expect a brief delay between the cellular post-ischemic oxidative burst and that originating from leukocytes. This delay helps to reconcile the temporal difference in post-ischemic $O_2^{-}$ generation between isolated heart and intact animal models. The contribution of $O_2^{-}$ generation from phagocytes during CABG with CPB is more difficult to assess. The compliment system is activated during CPB\(^{131}\), but also during off pump CABG\(^{132}\). Furthermore, while off-pump CABG is generally associated with decreased inflammation\(^{133-136}\), several studies indicate that the effect of the surgical procedure itself far outweighs the use of CPB as a pro-inflammatory factor during CABG\(^{137-139}\). Additionally, intraoperative reductions in inflammation do not necessarily reduce acute postoperative inflammation\(^{140}\).

More specifically, the question of whether polymorphonuclear leukocytes play an active role in mediating post-ischemic myocardial injury remains controversial. Inflammatory mediators are clearly activated and recruited to the post-ischemic myocardium, but neutrophil depletion does not appear to inhibit myocardial stunning and studies of clinical anti-neutrophil therapies do not generally produce clear benefits (reviewed in\(^{141}\)). Furthermore, superoxide dismutase scavenging of extracellular $O_2^{-}$ during ischemia and reperfusion has long been known to be ineffective at reducing myocardial ischemia-reperfusion injury\(^{142}\), while intracellular superoxide dismutase is more effective\(^{143, 144}\). These results indicate that endogenous intracellular free-radical generation is the central mediator of injury during ischemia and early reperfusion. These results support the concept that while the immune system plays a significant role in the
pathophysiology of ischemia-reperfusion injury; inflammation appears to be the consequence rather than the cause of myocardial injury.

1.4. Nitric oxide generation during myocardial ischemia and reperfusion

Since its identification as “endothelial derived relaxation factor”\textsuperscript{145-147}, several important roles spanning a host of physiological processes have been ascribed to nitric oxide (NO’). These include vasoregulation\textsuperscript{148}, neurotransmission\textsuperscript{149}, immunomodulation and defence\textsuperscript{150,151}, and cell signaling\textsuperscript{152}. The diverse physiological importance of NO’ belies its relatively recent discovery, but underscores its acclamation in 1992 by Science Magazine as “molecule of the year”\textsuperscript{153,154}.

NO’ is a relatively stable free radical whose half-life of a few seconds, combined with its solubility in both aqueous and organic solutions, allow it to freely diffuse across cell membranes. It spontaneously reacts with oxygen and water to form nitrite (equation 9), and is further oxidized in the presence of oxyhemoproteins to nitrate (equation 10a/b\textsuperscript{155}). Although dietary nitrates significantly contribute to their concentration in plasma, the concentrations of nitrate and nitrite after several hours of fasting, is a good indicator of overall NO’ activity\textsuperscript{156}.

\begin{equation}
4NO' + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (\text{Equation } 9)
\end{equation}

\begin{equation}
2RFe^{2+}O_2 + 3NO_2^- + 2H^+ \rightarrow 2Fe^{3+} + 3NO_3^- + H_2O \\
4RFe^{2+}O_2 + 4NO_2^- + 4H^+ \rightarrow 4Fe^{3+} + 4NO_3^- + O_2 + 2H_2O \quad (\text{Equation } 10a/b)
\end{equation}

NO’ is important in the context of myocardial ischemia-reperfusion injury primarily because it reacts with $O_2•$ to produce the peroxynitrite anion (ONOO’\textsuperscript{157}) (equation 11), which is itself far more reactive and toxic to biomolecules than its reactants. The reaction between $O_2•$ and NO’ is diffusion limited, and since the rate of ONOO’ formation
depends on the product of its reactant concentrations, small increases in O$_2^-$ or NO’ can rapidly increase ONOO’ levels.

Equation 11: \[ NO' + O_2' \overset{k=7 \times 10^9 M^{-1}s^{-1}}{\longrightarrow} ONOO' \]

When Zweier et al reported electron paramagnetic resonance spectra during ischemia and reperfusion, they noticed that the pattern of nitrogen-centered radical generation mirrored that of the oxygen-centered radical during ischemia and early reperfusion$^{57}$. Its uncertain identity was subsequently assumed to be a peroxylamine radical product of free-radical mediated peptide bond breakage$^{78}$. Similarly obtained electron spin resonance spectra demonstrated nitroxy radical adducts$^{59}$. The benefit of more recent findings suggests that this signal could have indicated the generation NO’ and/or nitrogen dioxide radical (NO$_2^+$), the latter being a breakdown product of peroxynitrous acid (ONOOH) (equation 17). Myocardial NO’ generation during both ischemia and reperfusion is a prerequisite for both cases. There are two likely sources of myocardial NO’ generation during ischemia and reperfusion; nitric oxide synthase (NOS) activity and nitrite reduction.

1.4.1 Nitric oxide synthases

The enzyme-catalyzed 5-electron oxidation of L-arginine to L-citrulline is the best studied NO’ producing biosynthetic pathway (equation 12). This reaction is catalyzed by one of three NOS enzyme isoforms; neuronal-, inducible-, and endothelial-NOS (nNOS, iNOS, and eNOS, respectively)$^{158}$. The myocardium constitutively expresses both eNOS and nNOS$^{159-161}$, and can be induced to express iNOS$^{161,162}$.

Equation 12: \[ 2LArg + 3NADPH + 3H^+ + 4O_2 \longrightarrow 2LCit + 3NADP^+ + 2NO' + 4H_2O \]
Enzymatically active NOS exist as a homodimeric oxidoreductase associated with the cofactor tetrahydrobiopterin, and contains heme, flavin adenine dinucleotide, and flavin mononucleotide moieties. Electron flux through all NOS isoforms requires calmodulin binding, but only in constitutive NOS isoforms (nNOS and eNOS) does calmodulin binding occur with calcium dependence\(^{163}\). Since L-arginine is the common substrate for enzymatic NO’ production, the pharmacology of several of L-arginine analogs, namely L-NMMA (\(N^G\)-monomethyl-L-arginine) and L-NAME (\(N^G\)-nitro-L-arginine methyl ester) were studied and identified as potent NOS inhibitors\(^{164}\).

 Constitutive NOS expressed in various cell types of the myocardium are a constant source of low-level NO’ generation in the normally perfused heart\(^{165}\). During ischemia, several factors that directly influence constitutive NOS activity are altered; intracellular calcium loading may by itself promote constitutive NOS activity, but decreased oxygen tension would conversely decrease enzyme activity. The restoration of oxygen tension at reperfusion in a calcium-loaded cell would by extension be expected to result in a sudden increase in NOS activity, assuming enzyme integrity was preserved throughout ischemia.

 This question was partly resolved using of electron paramagnetic resonance techniques capable of analyzing NO’, by the same group that used the same technique to definitively identify and characterize \(O_2^-\) production during ischemia and reperfusion one decade earlier\(^{166}\). These experiments, and similar studies clearly showed that NO’ is generated throughout the myocardium during ischemia, the majority of it being a product of NOS activity\(^{166-168}\). NO’ production was subsequently also shown to be increased at reperfusion, and here too the increase was largely dependent on NOS activity\(^{169}\). This result is somewhat expected because endothelial cells increase NO’ release in response to
shear stress through elevated eNOS activity (reviewed in\textsuperscript{170}), and shear stress increases in the coronary vasculature as a physical consequence of reperfusion. Paradoxically, the increased NO\textsuperscript{\textbullet} production at reperfusion also increases ONOO\textsuperscript{\textbullet} generation (equation 11), which irreversibly damages the heme domain of eNOS and oxidizes its tetrahydrobiopterin cofactor\textsuperscript{171}.

Although these results clearly demonstrate that myocardial NOS actively produces NO\textsuperscript{\textbullet} during ischemia and reperfusion, a parallel line of evidence shows that the enzyme is not unaffected by ischemia. The intracellular acidosis that accompanies prolonged ischemia can inhibit eNOS activity in a manner that, depending on the severity and duration of the acidosis, can be either reversible or irreversible\textsuperscript{172}. Interestingly, the heme domain of nNOS isolated from brain tissue was shown to be capable of producing O\textsubscript{2}\textsuperscript{\textsuperscript{\textbullet}} under depleted L-arginine concentrations\textsuperscript{173, 174}. Similar results were subsequently reported for eNOS\textsuperscript{175, 176} and the reductase domain of iNOS\textsuperscript{177, 178}. The conditions under which constitutive NOS isoforms shift to O\textsubscript{2}\textsuperscript{\textsuperscript{\textbullet}} production are now recognized to be dependent not only on the intracellular L-arginine supply, but also on tetrahydrobiopterin oxidation, which is a major consequence of prolonged ischemia\textsuperscript{179, 180}. In this way, uncoupled myocardial NOS can also contribute to O\textsubscript{2}\textsuperscript{\textsuperscript{\textbullet}} generation during ischemia and early reperfusion (Figure 2).

1.4.2 Nitrite reduction

The evidence that intracellular acidosis denatures and uncouples NOS, combined with the decreased O\textsubscript{2} availability as a NOS substrate appears to run counter to the observed increase in NO\textsuperscript{\textbullet} generated during ischemia. Furthermore, careful inspection of electron paramagnetic resonance results indicate that NO\textsuperscript{\textbullet} generation increases with prolonged
ischemia, and is not abolished under conditions of complete NOS inhibition\textsuperscript{166}. These results indicate the activity of a NOS-independent cellular NO$^\cdot$ source. This second source was identified as the reduction of nitrite (equation 13), which occurs physiologically in a non-enzymatic, pH-dependent fashion, when the cell exists in a sufficiently hypoxic and reduced state\textsuperscript{181}.

Equation 13: \[ 2NO_2^- + 4H^+ \leftrightarrow 2H_2O + 2NO^\cdot \]

In contrast to the L-arginine-NOS pathway of NO$^\cdot$ production, nitrite represents a reservoir for NO$^\cdot$ production during both ischemia and early reperfusion, when NOS enzymes can no longer be relied upon. Equation 13 implies that under sufficiently reductive and acidic conditions during ischemia, nitrites continuously feed chemical NO$^\cdot$ production. This reaction appears to proceed within the cellular context of myocardial ischemia only when the pH dropped below 6\textsuperscript{181}. If severe ischemic conditions can permanently denature a fraction of the myocardial NOS pool, and intracellular pH recovers sharply at reperfusion\textsuperscript{182}, then nitrite must be reduced to NO$^\cdot$ by an alternative mechanism in the early phase of reperfusion. One of these alternatives is mediated in blood under low oxygen tension by deoxygenated hemoglobin\textsuperscript{183}. This mechanism is presumed to induce vasodilation in hypoxic tissues, and may therefore be more relevant in blood circulating through ischemic tissues. Because the product of NO$^\cdot$ degradation also serves as its non-enzymatic generation, non-enzymatic nitrite reduction does not affect overall plasma nitrite and nitrate levels.

A second alternative pathway is mediated by xanthine oxidase. This enzyme was described as a source of nitrite reduction under zero-flow ischemia\textsuperscript{184-188}, where nitrite and O$_2$ compete for the O$_2$\textsuperscript{• -} generating mechanism illustrated by equation 7. Xanthine
oxidase can also catalyze nitrite reduction under the aerobic conditions encountered at reperfusion \(^{189}\). NO’ produced by the myocardium during ischemia and reperfusion likely represents the sum of the mechanisms described above: During ischemia, nitrite reduction—direct and/or xanthine oxidase mediated, depending on the degree of intracellular acidosis—produces NO’ in conjunction with functional NOS when they encounter substrate. At reperfusion, a burst of NO’ synthesis is driven by the fraction of non-denatured NOS enzymes, activated both by the shear stress that reperfusion imposes on the vascular endothelium and by intracellular Ca\(^{2+}\) overload with the influx of O\(_2\) substrate, complemented by nitrites that are in competition with O\(_2\) for xanthine oxidase derived electrons.

1.5. **Hydroxyl radical generation during myocardial ischemia and reperfusion**

The ability of exogenous superoxide dismutase to reduce ischemia-reperfusion injury established O\(_2^{-}\) as a harmful radical well before its generation could directly be measured in the myocardium. By contrast, O\(_2^{-}\) has a relatively benign reactivity towards biomolecules insofar as free radicals are concerned\(^{190-193}\). Furthermore, cellular antioxidant defenses convert O\(_2^{-}\) to O\(_2\) and H\(_2\)O at rates that approach diffusion limits (equations 3, 4, 5). These contradictory findings can be reconciled by a secondary O\(_2^{-}\) reaction, capable of competing with superoxide dismutase, which becomes more favorable during ischemia and reperfusion, which yields a product with more reactivity than its reactants.

The reactivity of the hydroxyl radical made it an obvious candidate as the end effector of oxidative cellular damage, and its definitive identification in the post-ischemic reperfused myocardium cemented this notion\(^{62, 78, 194, 195}\). Furthermore, the finding that its
signal was abolished by superoxide dismutase validated the concept that $O_2^\cdot-$, through some reaction within the cell, was a reactant in 'OH generation. Two pathways have been identified by which $O_2^\cdot-$ reactions can yield 'OH: iron-catalyzed reduction and ONOO$^-$/ONOOH decomposition.

1.5.1 Iron-catalyzed hydroxyl formation

The ability of transition metal ions to assume multiple oxidation states underscores their central role as catalysts for a host of cellular redox reactions. One such metal ion is iron. Biologically complexed iron can stably undergo single electron redox reactions that are not only critical to mitochondrial electron transport, but to cell function in general$^{196}$. This same attribute can also facilitate $Fe^{2+}$-mediated 'OH formation through the Fenton reaction (equation 14). In order for the Fenton reaction to proceed past one cycle, $Fe^{3+}$ must be reduced back to $Fe^{2+}$, and it is in this capacity (equation 15) that $O_2^\cdot-$ serves to perpetuate the Fenton reaction. The sum of the Fenton reaction and the superoxide-mediated reduction of $Fe^{3+}$ results in a complete redox reaction known as the Haber-Weiss reaction (equation 16)$^{197}$.

Equation 14:  
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

Equation 15:  
$$O_2^\cdot - + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

Equation 16:  
$$O_2^\cdot - + H_2O_2 \quad \! ^{k=1000M^{-1}s^{-1}} \! \rightarrow O_2 + \cdot OH + OH^-$$

Iron-catalyzed 'OH generation is problematic on several fronts. First, ferric iron precipitates in aqueous solutions at physiological pH, while ferrous iron, despite being soluble, reduces $O_2$ to produce ferric iron and $O_2^\cdot -$. Virtually all physiological iron consequently exists as heme, or protein complexes, leaving only trace amounts of non-
chelated iron$^{198}$. This limitation can be overcome because $O_2^{-}$ is known to destabilize several iron-sulfur containing enzymes, liberating $Fe^{2+}$ in the process to catalyze the Fenton reaction$^{199}$. Even if the extent to which this happens is small, the overall Haber-Weiss reaction can proceed at very low $H_2O_2$ and iron concentrations with rate constant of about $1000 \text{ M}^{-1}\text{•s}^{-1}$, provided adequate $O_2^{-}$ can be supplied$^{200}$.

Another problem with iron-catalyzed 'OH generation results from its relatively low kinetic rate constant. Not only does the reaction between $O_2^{-}$ and superoxide dismutase have a rate constant in excess of five orders of magnitude greater than that estimated for the Haber-Weiss reaction, the high concentration of superoxide dismutase dictates that $O_2^{-}$ is much more likely to encounter superoxide dismutase than cellular $Fe^{2+}$. Catalase-mediated $H_2O_2$ oxidation is also more likely than $H_2O_2$ reduction in the Fenton reaction for similar reasons$^{201}$. Despite these limitations, the finding that the iron chelator desferrioxamine can reduce ischemia-reperfusion injury$^{202-204}$ (although not universally so$^{205, 206}$) lends support to the notion that cellular $Fe^{2+}$ can contribute to 'OH generation during ischemia and reperfusion.

1.5.2  Peroxynitrous acid degradation

The extent to which trace metals catalyze 'OH generation is unresolved. The kinetics of the overall Haber-Weiss reaction in a superoxide dismutase - and catalase-containing environment makes it an unlikely pathway for the increases in 'OH generation during myocardial ischemia and reperfusion. The alternative source of 'OH must be able to sufficiently compete with the kinetics and cellular concentration of superoxide dismutase. To this end, the reaction between $O_2^{-}$ and NO' which yields ONOO' (equation 11)$^{157}$
occurs spontaneously and with a rate constant that, like those of superoxide dismutase and catalase, approaches diffusion$^{207, 208}$. 

ONOO$^-$ is relatively stable and has a 1.9 second half-life at pH 7.4, which permits diffusion over a distance of several cell diameters, presumably through anion channels$^{209}$. Conversely, ONOOH is a strong oxidizing agent that is inherently unstable. ONOOH can rapidly decompose to produce NO$_2^-$ and ‘OH radicals$^{209}$ (equation 17). The pK$_a$ of the ONOO$^-$/ONOOH acid-base pair is 6.8 at $37^\circ$C$^{210}$, so ONOO$^-$ becomes fractionally more protonated with increasing intracellular acidification during ischemia. The decay of ONOOH is an attractive model for ‘OH generation because it does not depend on the Fenton reaction, is not inhibited by catalase activity$^{211}$, and preferentially yields ‘OH during conditions of ischemia and reperfusion.

Equation 17: \[ \text{ONOO}^- + H^+ \xrightarrow{pK_a=6.8} \text{ONOOH} \rightarrow \text{NO}_2^- + \text{‘OH} \]

Despite the fact that myocardial generation of O$_2^-$ and NO’ are not restricted to ischemia and reperfusion, the generation of ONOO$^-$ and ‘OH are not appreciable until the process has taken place$^{78, 124, 169}$. The likelihood that O$_2^-$ will react with superoxide dismutase or NO’ depends on their relative cellular concentrations. The concentration of superoxide dismutase in the normally functioning myocardium exceeds that of NO’ by several orders of magnitude, so equation 11 is unlikely to occur and very little ONOO$^-$ will be generated. The concentration of NO’ approaches and surpasses that of superoxide dismutase during reperfusion, and equation 11 becomes the dominant reaction$^{209, 212}$. The finding that exogenous superoxide dismutase can decrease myocardial ‘OH generation further reflects the notion that a balance exists between equation 3 and equation 11 as the dominant O$_2^-$ reaction.
The relative contribution of ONOO-/ONOOH degradation and the Haber-Weiss reaction to •OH generation during ischemia and reperfusion remains a question of debate. The rate constant of the Haber-Weiss reaction is low in comparison to that of ONOO• generation, but myocardial H₂O₂ content can dramatically increase during ischemia and reperfusion²¹³ and both desferrioxamine and catalase can reduce post-ischemic myocardial •OH generation²¹⁴. Furthermore, lipid peroxidation products have somewhat paradoxically been found to directly correlate with superoxide dismutase and H₂O₂ concentrations, while chelators and •OH scavengers appear break this relationship²¹⁵. On the other hand, the protective effects of desferrioxamine have partly been ascribed to its ability to inhibit ONOO•-mediated oxidation reactions rather than iron chelation²⁰⁹,²¹⁰ and catalase activity does not necessarily reduce infarct size²¹⁶,²¹⁷.

\[
NO• + O₂•- \overset{k=7.2 \times 10^9 M^{-1} s^{-1}}{\longrightarrow} ONOO• + H^+ \overset{bK_a=6.8}{\longrightarrow} ONOOH
\]

**Figure 3**: Main hydroxyl radical producing pathways during myocardial ischemia and reperfusion.
The loss of a protective effect at higher doses of superoxide dismutase has also been attributed to an increase in H2O2 production by superoxide dismutase, which in turn inhibits superoxide dismutase activity and drives 'OH generation through the Fenton reaction. In support of this notion, covalent superoxide dismutase-catalase conjugates can inhibit 'OH generation and reduce ischemia-reperfusion injury at concentrations that induce toxicity with non-conjugated superoxide dismutase. Several mathematical models contest the notion that superoxide dismutase influences cellular H2O2 levels unless a large proportion of O2•− is eliminated from the system by a mechanism that is independent of superoxide dismutase. To this end, O2•− can be rapidly eliminated by reacting with NO• during ischemia and reperfusion. Thus, O2•− derived 'OH radicals likely result from ONOO•/ONOOH degradation proceeding in concert with the Haber-Weiss reaction during ischemia and reperfusion (Figure 3).

1.6. Free radical-mediated damage during myocardial ischemia and reperfusion

The myocardium functions as a large interconnected electrical circuit in which synchronized diastole-systole cycling and contraction rely on coordinated conductivity during its component cardiomyocytes. Impaired heart function subsequent to ischemia and reperfusion is by extension a symptom of cell damage that regionally uncouples the myocardial conductivity network. The severity of cell damage incurred, which ranges from reversible to irreversible, is proportional to the magnitude of ROS reactions within cardiomyocytes.

Radical reactions are stepwise, spontaneous, self-propagating chain reactions. Free radical propagation reactions in the context of biological injury primarily result in hydrogen abstraction from alkene groups. Since these groups occur in many, but not all
biomolecules, these reactions are semi-random in nature. From the perspective of the cell, the semi-random uncontrolled nature of radical reactions makes them inherently damaging. Free radicals are generally strong oxidants, but the degree to which they exert these reactions depends on their individual reactivity towards the biomolecules that they encounter. For example, the short half-life and reactivity of the \( \cdot \)OH radical dictates that it abstracts hydrogen atoms from a wide array of biomolecules provided they are located in the cellular vicinity of the \( \cdot \)OH radical at the time of its generation. Alternatively, the less reactive \( O_2^- \) and \( NO^- \) radicals can diffuse to more distal locations from their origins prior to mediating radical reactions on more specific targets.

The first phase of radical reactions is an initiation that generates the original radical. In the context of ischemia-reperfusion injury, initiation reactions are those that produce \( O_2^- \), \( NO^- \), and \( \cdot \)OH. These processes have been discussed in detail within the context of myocardial ischemia and reperfusion in sections 1.2, 1.3, 1.4, and 1.5.

The second phase is a series of propagation reactions. These include substitution reactions that often involve hydrogen atom abstraction from an alkene group to produce an alkyl radical, and addition reactions exemplified by the integration of molecular oxygen to the alkyl radical to yield a peroxyl radical. The reaction is further propagated because any reaction involving a radical and a non-radical invariably yields a free-radical product. The aforementioned peroxyl radical, for example, can abstract a hydrogen atom from another alkene to yield a hydroperoxide and a new alkyl radical.

The final phase of radical reactions is the termination step, which occurs when a radical undergoes a second single-electron redox reaction to produce a terminal non-radical. The products of radical reactions are non-enzymatically modified organic
biomolecules that are usually disruptive to the function of the larger cell structures in which they occur. Thus, lipid peroxidation products can compromise the integrity of cell membranes or of proteins embedded therein; amino acid nitration or oxidation can impair normal protein function; and DNA oxidation is mutagenic.

The semi-random nature of radical reactions does not necessarily yield irreversible cell damage: if non-critical proteins are only fractionally damaged, or if the magnitude of membrane, protein, or DNA damage does not adversely affect function, then the cell will survive. In this case, normal cell processes such as phospholipid and protein turnover can remove the altered biomolecule. Alternatively, radical reactions can denature or activate critical biomolecules to initiate pro-apoptotic processes. In the most severe cases, when radical reactions compromise critical cell functions and/or membrane integrity, the cell becomes necrotic. The cellular consequence of radical reactions within the myocardium is therefore a question of scale and radical concentration, and a question of the probability that certain critical cellular components will be affected.

1.6.1 Lipid peroxidation

Phospholipid bilayers form the biological membranes that encapsulate cells and their organelles. The balance between membrane integrity and fluidity depend on their constituent saturated and unsaturated fatty acid components. The alkenes in unsaturated fatty acids are susceptible to hydrogen abstraction by ROS to yield carbon-centered and peroxyl radicals. These are the carbon- and oxygen-centered lipid radicals that were observed in early paramagnetic resonance studies and were first definitively identified as such by Arroyo et al in 1987. The propagation phase of lipid peroxidation is effectively an amplification of the initial oxygen radical mediated hydrogen abstraction that
continues until two radical species combine in a termination reaction. Interestingly, NO’
is not reactive enough to initiate lipid peroxidation, but it can act as a chain terminator\textsuperscript{227}. Peroxyl and lipid isoforms and can react within and between one another to produce a heterogeneous array of end products that encompass isoprostane-like compounds and acid-mediated fragmentation products that include malondialdehyde (MDA) (Figure 4)\textsuperscript{228-231}. These altered fatty acids can adversely affect the functional integrity of lipid membranes, and are therefore quickly cleaved and released by phospholipases\textsuperscript{232}. 
Figure 4: Generalized example scheme of lipid peroxidation. X and Y represent independent chain lengths and structures. Note that hydrogen abstraction can occur with similar frequency at other regions within a given precursor structure, that additions can occur at a variety of resonance structures, and that “isoprostane like compounds” represent a host of compounds with a host of structures (loosely adapted from$^{233}$ and$^{230}$).
In vivo free radical reactions produce a host of compounds that are not otherwise catalyzed by the cell. The non-enzymatic catalyzed origin of these compounds makes them potentially useful markers of oxidative stress provided they can be accurately and quantitatively analyzed. To this end, multi-center studies of various non-invasive biomarkers of oxidative stress specifically identified two lipid-peroxidation products, MDA and isoprostanes, as indicators of increased free radical activity\textsuperscript{234-236}. MDA and isoprostane levels are known to increase during myocardial ischemia and reperfusion\textsuperscript{237-240}, one isoprostane isomer, 15-F\textsubscript{2t}-isoprostane, induces dose-dependent vasoconstriction in the coronary vasculature\textsuperscript{241,242}. As such, 15-F\textsubscript{2t}-isoprostane formation may exacerbate or prolong cardiac dysfunction after ischemia and reperfusion\textsuperscript{243-245}. From a clinical perspective, 15-F\textsubscript{2t}-isoprostane levels increase during coronary angioplasty and coronary artery bypass grafting procedures\textsuperscript{246-248}, and it has been associated with a decline in postoperative cardiac function\textsuperscript{244,245,249}.

Unsaturated fatty acids in the mitochondrial membrane are especially vulnerable to peroxidation because of their intimate proximity to ROS generated by complex III during myocardial ischemia and reperfusion. One particular mitochondrial lipid, cardiolipin, constitutes nearly 20\% of the inner membrane\textsuperscript{250}, and is a key regulator of mitochondrial integrity and function\textsuperscript{251}. Specifically, electron transport through cytochrome c oxidase depends on its association with cardiolipin\textsuperscript{252,253}. Cardiolipin content decreases with increased lipid peroxidation during myocardial ischemia\textsuperscript{81,82}, which appears to facilitate cytochrome c release and apoptotic signaling during reperfusion\textsuperscript{254} (reviewed in\textsuperscript{255}). These alterations coincide with and result in the inhibition of cytochrome c oxidase activity\textsuperscript{256,257}, and may ultimately also inhibit post-ischemic electron flux through
complex I\textsuperscript{258} and complex III\textsuperscript{259}. Cardiolipin depletion and cytochrome c release can be preserved if complex I of the electron transport chain is pharmacologically inhibited prior to ischemia\textsuperscript{260}. These results further underscore the central role that mitochondria play in the pathological ROS generation during myocardial ischemia-reperfusion injury.

1.6.2 Protein oxidation and nitration

The alkene groups of unsaturated fatty acids in cell membranes are prone to hydrogen abstraction, but ROS can induce cardiomyocyte apoptosis and necrosis even when cellular and subcellular membranes remain functionally intact. This occurs because cellular proteins can be non-enzymatically modified by ROS. Amino acid oxidation and nitration within proteins underlies several clinical ischemia-reperfusion injury pathologies. Specifically, the activation of necrosis and pro-apoptotic proteins underlies infarction\textsuperscript{261} while damaged contractile proteins impairs contraction to yield myocardial stunning\textsuperscript{262-266}.

Proteins are susceptible to ROS damage because the functional groups and backbone \( \alpha \)-carbons of all amino acids are susceptible to hydrogen abstraction by \( \cdot \text{OH} \). The nature of these oxidation reactions and the products that they produce is a function of the amino acid functional group, the type of ROS, and the semi-random nature of radical reactions (Figure 5)\textsuperscript{267-271}. A complete catalogue of the potential reactions would have to account for post-translationally modified amino acids, the presence or absence of adjacent functional groups, and reactions between amino acids and non-protein radicals such as lipid radicals.
Figure 5: Generalized examples of free-radical mediated amino acid modification scheme. *Upper panel:* ‘OH -mediated hydrogen abstraction from tertiary carbons within an amino acid or peptide. *Middle panel:* ‘OH -mediated hydrogen abstraction or ONOOH-mediated oxidation of the aromatic group of phenolic amino acid. *Lower panel:* ‘OH-mediated hydrogen abstraction from a sulphydryl or methionine amino acid. R represents a generalized continued amino acid or peptide motif.
Free radical oxidation reactions can denature proteins by cleaving peptide bonds, by cross-linking functional groups, and by altering the hydrophobicity of amino acids on protein surfaces\textsuperscript{267, 272}. However, not all of these reactions are irreversible; methionine oxidation is enzymatically reduced back to methionine in a system that may contribute to cellular antioxidant defences\textsuperscript{273, 274}. Disulfide bonds can likewise be enzymatically reduced\textsuperscript{275}. Conversely, H\textsubscript{2}O\textsubscript{2} generated during ischemia by the increasing activity of superoxide dismutase can deplete the glutathione antioxidant system. In this vein, cellular and mitochondrial sulfhydryl groups are significantly oxidized after ischemia and reperfusion, while tyrosine dimerization and protein-lipid peroxide conjugation products increase\textsuperscript{276}. Oxidation of regulatory sulfhydryl groups in mitochondrial complex II markedly reduces electron transport in rat hearts following ischemia and reperfusion\textsuperscript{277}. The ONOO\textsuperscript{−}-mediated oxidation of sulfhydryl groups proceeds with a rate constant that exceeds that of H\textsubscript{2}O\textsubscript{2} by three orders of magnitude\textsuperscript{278}, and its generation is thus capable of rapidly depleting endogenous antioxidant stores.

Mass spectrometry proteomic techniques applied to myocardial biopsies enable the analysis of protein changes during ischemia and reperfusion\textsuperscript{279}. The use of these techniques in isolated heart models indicates that a variety of myocardial proteins are physically and chemically modified during ischemia and early reperfusion rather than differentially expressed\textsuperscript{280}. More comprehensive studies revealed that protein modifications during ischemia involve several ROS-independent and a limited number or ROS-dependent modifications, while damage during reperfusion is primarily ROS-dependent and is a major cause of post-reperfusion myocardial contractile dysfunction\textsuperscript{281}. 

\textsuperscript{267, 272}
These findings support the notion that ischemic ROS generation depletes endogenous antioxidant defenses and prime the cell for ROS damage at reperfusion.

The phenolic groups of tyrosine residues are the functional units for a wide variety of cell signaling proteins collectively called tyrosine kinases and protein tyrosine phosphatases. The integrity of these signaling proteins and the signal transduction pathways that they encompass are vulnerable to ONOO\(^{-}\)-mediated oxidation reactions. These tyrosine oxidation reactions can yield dityrosine and 3-nitrotyrosine\(^{282-284}\). Accordingly, myocardial 3-nitrotyrosine and dityrosine content increases after ischemia and reperfusion\(^{124,169,212,285}\). Proteomic analysis subsequent to ischemia and reperfusion revealed that mitochondrial proteins were particularly at risk of tyrosine nitration\(^{286}\), reflecting their proximity to the cellular ONOO\(^{-}\) source\(^{287}\). To this end, nitration and oxidation of tyrosine and cysteine residues within mitochondrial complex II at reperfusion is one mechanism by which decreased mitochondrial respiration persists despite the recovery of myocardial oxygen tension\(^{288,289}\).

1.6.3 DNA damage

DNA molecules are susceptible to ROS damage in the same basic way that proteins are, namely, an initial \(^{\cdot}OH\)-mediated hydrogen abstraction followed by purine or pyrimidine modification and/or fragmentation\(^{290}\). Interestingly, MDA, an ROS-mediated lipid peroxidation product, is a carcinogen because it has the ability to form adducts with DNA bases\(^{291}\). In this way, ROS-mediated lipid peroxidation can both directly and indirectly damage DNA. Genomic damage can initiate apoptotic pathways and impair myocardial protein expression. The initiation of these processes can deplete myocardial energy and NAD\(^{+}\) stores, and can impair repletion of cellular energy stores by inhibiting
glycolysis and mitochondrial function\textsuperscript{292}. On the other hand, apoptotic cascades are more likely to result from protein or cell membrane damage than from DNA damage, and ROS-mediated genetic mutations are not generally considered hallmarks of myocardial ischemia-reperfusion injury.

1.6.4 Matrix metalloproteinases

Myocardial regions that are subject to prolonged sub-lethal ischemia undergo structural rearrangements that are characteristic of the hibernating myocardium. This myocardial remodeling proceeds in part on account of extracellular matrix metalloproteinase (MMP) activity. MMPs can be transcriptionally upregulated under conditions of chronic tissue remodeling, but they are also acutely activated inside the cell by ONOO\textsuperscript{−} or H\textsubscript{2}O\textsubscript{2} mediated proenzyme cleavage\textsuperscript{293-297}. Accordingly, MMP activity can contribute to ischemia-reperfusion injury in which post-operative myocardial function is depressed despite the absence of appreciable apoptosis and necrosis. During cardiac surgery, ischemia-reperfusion injury often manifests itself by persistently reduced contractility in the absence of large-scale myocyte loss. This post-operative phenomenon is called myocardial stunning, and is a major contributing factor to post-operative low cardiac output syndrome. The impaired contractility of individual myocytes is in part the result of MMP-2-mediated degradation of intracellular proteins including myosin light chain\textsuperscript{298}, \(\alpha\)-actinin\textsuperscript{299}, and troponin I\textsuperscript{300,301}. From the perspective of cardiac surgery, ischemia and reperfusion incurred during CABG with CPB activates both MMP-9 and MMP-2 inside the cell where they digest intracellular contractile target proteins. This activation inversely correlates with postoperative cardiac function\textsuperscript{302-304}. By extension, ONOO\textsuperscript{−} -mediated contractile dysfunction in the isolated cardiomyocyte can be decreased
by pharmacologically inhibiting MMP activity\textsuperscript{305}. Furthermore, MMP inhibition decreases endothelial injury and improves myocardial functional recovery in an isolated heart model of ischemia-reperfusion injury\textsuperscript{300,306}. Thus, from the perspective of contractile proteins, ROS generated during ischemia and reperfusion can persistently impair myocardial function by non-enzymatic protein degradation and modification, as well as by activating specific proteolytic enzymes.

1.6.5 Mitochondrial permeability transition

Large-scale mitochondrial ROS generation during ischemia and reperfusion induces a cellular process called mitochondrial permeability transition\textsuperscript{307-313} (Figure 6). This process is strongly inhibited during ischemia by intracellular acidosis below pH 7, but favored by depleted energy stores\textsuperscript{313,314} and increased calcium loading\textsuperscript{314}. Accordingly, mitochondrial permeability transition takes place during the first few minutes of reperfusion when intracellular pH starts to recover\textsuperscript{315-318}. 
Figure 6: Summary of the major processes and consequences of mitochondrial permeability transition. *(ROS=reactive oxygen species; Casp=Caspase; Apaf=apoptotic peptidase activation factor; $\Psi_m$=inner mitochondrial membrane potential).*
Mitochondrial permeability transition involves the formation and opening of pores on the inner mitochondrial membrane that allow nonspecific conductance of molecules smaller that 1.5kDa in size\textsuperscript{319,320}. Mitochondrial permeability transition rapidly uncouples oxidative phosphorylation, dissipates the mitochondrial membrane potential, and further depletes cellular ATP stores. Matrix proteins, generally being too large to pass through the pores, exert a colloidosmotic pressure that causes the mitochondrial matrix to swell, thus compromising the integrity of the outer membrane. Simultaneous cardiolipin peroxidation in the inner membrane liberates cytochrome c\textsuperscript{321,322}, which can subsequently be released to the cytoplasm where it initiates strong pro-apoptotic signal cascades\textsuperscript{323}. The severity of mitochondrial permeability transition directly affects the ability of the cell to maintain ATP stores. If mitochondrial permeability transition is transient and the outer membrane remains intact, then the cell can recover and survive\textsuperscript{310}. Alternatively, a sufficiently energized cell will become apoptotic under sustained mitochondrial permeability transition. Lastly, sustained mitochondrial permeability transition in cells that have severely depleted ATP stores will become necrotic\textsuperscript{324,325}. Accordingly, the number of mitochondria undergoing mitochondrial permeability transition correlates with the likelihood of cardiomyocyte loss\textsuperscript{312} and the severity of mitochondrial permeability transition is inversely proportional to the recovery of contractile function in an isolated heart model\textsuperscript{326}. Postconditioning, a phenomenon in which protection is afforded by staggered intermittent reperfusion, appears to extend the mitochondrial permeability transition-inhibitory features of ischemia into the early phase of reperfusion\textsuperscript{327,328}.
1.7. Protective strategies during cardiac surgery

The prevalence of coronary artery disease, combined with the lethality of its manifestations account for a major cause of death and disability worldwide. Stable atherosclerotic plaques result in chronic ischemia, which in turn leads to myocardial stunning, hibernation and remodeling anterograde of the plaque. These changes result in heart failure, and require revascularization to restore adequate cardiac performance. More urgently, unstable atherosclerotic plaques can rupture to yield coronary artery occlusions that, unless treated with emergency revascularization, can result in fatal acute myocardial infarction (reviewed in\textsuperscript{329}). Revascularization by definition exposes the heart to ischemia-reperfusion injury, the magnitude of which directly impacts post-operative patient outcomes.

Strategies aimed at reducing ROS-mediated damage incurred during myocardial ischemia and reperfusion are generally targeted towards the three phases of free radical reaction progression. Thus, protection may involve inhibition or quenching of $\text{O}_2^\bullet-$, NO$^\bullet$, or $\text{OH}^\bullet$ generation during ischemia and early reperfusion. Alternatively increasing the likelihood that a stabile, less reactive free radical will be generated can promote chain termination reactions. These strategies are made inherently more complex by the important physiological –and even cardioprotective role that both $\text{O}_2^\bullet$ and NO$^\bullet$ play as signaling molecules, and by the clinical nature in which these strategies must ultimately be employed. Indeed, several such cardioprotective strategies have been developed, but translating laboratory-based successes to the clinical setting remains elusive\textsuperscript{330, 331}.

Despite these difficulties, several interventions are known to reduce the severity of ischemia-reperfusion injury, and do so by altering one or more phases of free-radical
reactions in the myocardium. Chief among these is ischemic preconditioning, which involves a host of endogenous signaling steps that ultimately reduce ROS generation at reperfusion. Volatile anesthetics can pharmacologically activate protective pathways that may be common to ischemic preconditioning, thus potentially making preconditioning more clinically accessible. Finally, the application of compounds with antioxidant capacities to supplement endogenous antioxidant systems has long been thought to inhibit the propagation phase and promote the termination phase of radical reactions.

1.7.1 Ischemic preconditioning

The roots of preconditioning stems from a study by Murry et al, who coined the term and were first to report that infarct size subsequent to a 40-minute circumflex occlusion was significantly smaller in canine hearts that had been preconditioned with four transient five-minute circumflex occlusions separated by five minutes of reperfusion, than in canine hearts in which the 40-minute occlusion was not preceded by a preconditioning stimulus. In a related paper, the same group demonstrated that four successive ten-minute coronary occlusions did not deplete myocardial adenine nucleotide pools beyond the first occlusion, and that the initial depletion was less severe than during a single, continuous 40-minute occlusion. Seven years earlier, Verdouw et al, investigating if pigs could serve as their own controls to study myocardial ischemia, noticed that the first ischemic episode induced a quantitatively different metabolic profile than the second ischemic episode. These authors speculated that the metabolomic difference resulted from myocardial glycogen and high-energy phosphate depletion, and/or necrosis, but failed to recognize that the first ischemic episode may have been inducing a cardioprotective state in anticipation of the second. In 1993, it was discovered that
ischemic preconditioning also induced longer-term changes that were protective between 12-72 h after the initial ischemic episode. These changes were found to be mediated, at least in part, by increasing the translation of stress proteins including HSP72 and HSP60. In the clinical setting, Kloner et al observed that patients presenting with acute myocardial infarctions who had any previous history of angina were less likely to suffer from severe congestive heart failure, in hospital death, shock, or a combination thereof relative to those patients with no previous history of angina.

Ischemic preconditioning is mechanistically complex and not completely understood. The end effectors of the early preconditioning involve post-translational differences to the myocardial proteome, while late preconditioning recruits transcriptional changes. Preconditioning in general is a complex mix of cell signaling and channel activation: the phenomenon appears to be predicated on the indirect activation of several key pro-survival pathways including the phosphatidylinositol-3-kinase-Akt and extracellular signal regulated kinase pathways, which in turn converge on key end-effectors. Chief among these are the ATP-sensitive potassium (\(K_{\text{ATP}}\)) channel. \(K_{\text{ATP}}\) channels are found on the membranes of mitochondria and sarcolemma, and become activated by low ATP concentrations. Mitochondrial \(K_{\text{ATP}}\) channels play an important physiological role in the maintenance of mitochondrial matrix volume during oxidative phosphorylation by regulating osmotic gradients. Preconditioning activates sarcolemmal \(K_{\text{ATP}}\) channels, allowing a net inward potassium current to the cytoplasm. This inward potassium current stabilizes the cell membrane potential, thereby decreasing \(\text{Ca}^{2+}\) influx. In turn, decreased \(\text{Ca}^{2+}\) loading is thought to reduce both contracture and the severity of the ROS burst. The activation of mitochondrial \(K_{\text{ATP}}\) channels also increases \(\text{O}_2^{-}\) generation at
complex I of the electron transport chain, which presumably initiates protective signaling cascades prior to ischemia and reperfusion\textsuperscript{341}.

Paradoxically, both O$_2^-$ and NO$^\cdot$ signaling play critical roles in ischemic preconditioning\textsuperscript{92,342-344} but neither ONOO$^-$ nor OH appear to be appreciably formed\textsuperscript{345,346}. Regardless of the role that ROS play in preconditioning signaling, ischemic preconditioning ultimately reduces the magnitude injurious ROS activity at reperfusion\textsuperscript{347}. Several possibilities could explain this apparent paradox. First, the ischemic episodes that induce a preconditioning effect are relatively short, so the magnitude with which NO$^\cdot$ is generated during ischemic preconditioning may be insufficient to effectively compete with superoxide dismutase-mediated O$_2^-$ dismutation. Thus ONOO$^-$ generation would be averted. Second, O$_2^-$ and NO$^\cdot$ generation may be temporally and/or spatially separated during ischemic preconditioning. Either of these possibilities would ultimately reduce the magnitude of ONOO$^-$ generation during the signaling phase of this phenomenon. In support of this, NO$^\cdot$ generation during ischemia reduces the magnitude of ROS generation during both ischemia and reperfusion\textsuperscript{348}, and ischemic preconditioning in turn relies on moderate O$_2^-$ increases in the absence of large scale NO$^\cdot$ generation, which does not effectively lend itself to ONOO$^-$ formation and thus reduces the likelihood of intracellular MMP activation\textsuperscript{349}. Ultimately, preconditioning reduces intracellular 3-nitrotyrosine staining and maintains electron transport chain coupling as evidenced by conserved NADH dehydrogenase and cytochrome oxidase activity subsequent to ischemia and reperfusion\textsuperscript{346}.

From a clinical perspective, ischemic preconditioning is restricted to those contexts in which prolonged ischemia can be anticipated. To this end, a host of studies have
investigated various clinical outcomes subsequent to ischemic preconditioning protocols within the context of CABG with CPB (reviewed in\textsuperscript{350,351}). Notably, analysis of excised biopsies revealed that preconditioning can preserve myocardial ATP stores during CABG\textsuperscript{352,353}. A systematic review and meta-analysis of clinical ischemic preconditioning studies in human subjects identified clear reductions in postoperative ventricular arrhythmias, inotropic requirements, and intensive care unit stay among patients receiving ischemic preconditioning protocols\textsuperscript{351}.

Ischemic preconditioning within the context of cardiac surgery necessitates the application of an invasive treatment to the heart in the form of repetitive aortic clamping and declamping, which has the potential to destabilize plaques and may increase the likelihood of thromboembolism, among other risks. Some of these risks may be reduced with other conditioning strategies such as ischemic postconditioning and remote ischemic conditioning. The impact of postconditioning and remote preconditioning on ROS formation in first few minutes of reperfusion are not as clear as that of ischemic preconditioning, and these conditioning strategies are extensively reviewed elsewhere\textsuperscript{354,355}. In the absence of clear evidence from a uniform large clinical trial speaking to its benefits with respect to overall post-operative mortality, clinicians will continue to be reluctant in adopting these strategies in the operative setting.

1.7.2 \textit{Volatile anesthetic preconditioning}

In 1983, Davis \textit{et al} reported that halothane anesthesia was capable of reducing infarct size in dogs undergoing coronary artery occlusion\textsuperscript{356}. Six years later, Davis and Sidi reported that isoflurane-anesthetized dogs had a decreased myocardial infarct size and reduced necrosis and O\textsubscript{2} consumption after left anterior descending coronary artery
occlusion, compared to control dogs. Still, it was not until 1997 that the capacity of anesthetics to induce a preconditioning-like effect was realized.

A variety of anesthetics appear to induce a preconditioning-like effect in a variety of animal and experimental models of ischemia-reperfusion injury (reviewed in). Like ischemic preconditioning, volatile anesthetics indirectly increase the open probability of $K_{ATP}$ channels. By contrast, volatile anesthetics appear to induce different gene and protein expression patterns than ischemic preconditioning. Whether these latter changes can be sufficiently induced and functional at reperfusion within the operative timeline is questionable.

Volatile anesthetic preconditioning is easily translated to the operative setting. To this end, several studies have been conducted in which volatile anesthetics were compared either with one another and/or with intravenous anesthetic, showing improvements in biochemical markers of myocardial damage, hemodynamic function, and hospital stay. Unfortunately, two notable multi-center, randomized trials failed to replicate decreases in troponin I with a sevoflurane preconditioning protocol, while a third study found a similar lack of troponin I reduction with an isoflurane protocol. To this end, the preconditioning protocol itself appears to influence its successful activation, and it has been argued that patient and operative factors may play an important role in whether or not volatile anesthetics confer additional benefits during cardiac surgery.

Of equal interest, a recent meta-analysis comparing sevoflurane to propofol anesthesia found that patients receiving sevoflurane generally had higher post-bypass cardiac indices, decreased serum troponin I, shorter intensive care unit and hospital stays, and lower incidence of myocardial ischemia. Notably, this meta analysis failed to
detect any significant differences in postoperative ventilation time, inotropic support, mortality, myocardial infarction, and atrial fibrillation\textsuperscript{382}. These latter results are in agreement with a previous retrospective review comparing sevoflurane to propofol anesthesia in 10,535 patients undergoing cardiac surgery. This study failed to detect any significant differences in 30 day mortality, post-operative myocardial infarction and arrhythmia\textsuperscript{383}. In a similar vein, a recent study comparing sevoflurane, isoflurane and propofol during CABG found that patients receiving volatile anesthesia had improved troponin I profiles while those receiving propofol had less lipid peroxidation, improved glutathione peroxidase activity, and decreased NO\textsuperscript{•} production\textsuperscript{384}. A more comprehensive study comparing isoflurane and propofol anesthesia failed to find any significant differences in postoperative troponin-I, hospital or intensive care unit stays, or in hospital-, 30-day- and 1-year cardiac morbidity and mortality\textsuperscript{385}.

1.7.3 Antioxidants

Chemical antioxidants can be characterized as compounds that inhibit the propagation step of free radical reactions. The antioxidant capacity of a compound depends on two critical factors: its solubility in the medium in which radical reactions are occurring and its ability to stabilize an unpaired electron. Antioxidant inhibition of the radical propagation phase necessarily involves a chemical reaction, which necessitates its interaction with a given reactive radical. Thus, if the radical reaction to be inhibited involves lipid peroxidation, it would be desirable if the antioxidant in question were lipophilic. The propagation phase is stalled when a relatively stable radical with lower reactivity is produced. The stability of a radical depends in part on the area across which the lone electron can be resonance-stabilized. Accordingly, large conjugated networks
can resonance-stabilize lone electrons throughout their conjugated polyene or aromatic Pi orbitals, and tertiary radicals are inherently more stable than secondary-, and in turn primary radicals\textsuperscript{386}. \(\beta\)-Carotene, for example, is an organic compound containing a network of 22 conjugated carbon atoms\textsuperscript{387}. Its antioxidant capacity is derived from its ability to stabilize an unpaired electron across a large conjugation network of 11 unsaturated bonds containing eight tertiary carbons subsequent to hydrogen abstraction\textsuperscript{388}.

Although the resonance stabilization of an unpaired electron is not a termination reaction, more stable radicals are more likely to react with more reactive radicals than with less reactive non-radicals. In the case of the \(\beta\)-carotene radical, reaction with a second peroxyl radical can yield a host of non-radical products, each corresponding to a possible resonance structure\textsuperscript{389}.

Antioxidant supplementation is a logical adjuvant therapy during CABG, because the operative procedure itself is associated with a depletion of intrinsic antioxidant capacity\textsuperscript{390}. Accordingly, several clinical studies have measured surrogate markers of oxidative stress and preliminary outcomes when various antioxidants have been supplied during CABG procedures. The first of these antioxidants is N-acetylcysteine, which reacts with \(^{\cdot}\)OH and to a lesser extent H\(_2\)O\(_2\), and is a physiological precursor to glutathione\textsuperscript{391}. The administration of N-acetylcysteine appears to effectively decrease MDA levels, but this decrease is not associated with improved troponin-I or myocardial creatine kinase (CK-MB) profiles\textsuperscript{392-395}, with few exceptions\textsuperscript{396}. Furthermore, while one comprehensive randomized, double-blind, placebo-controlled clinical trial found that N-acetylcysteine administered during CPB decreased 3-nitrotyrosine and 15-F\(_{2\alpha}\)-isoprostane
staining – albeit in the absence of hemodynamic and clinical outcome differences\textsuperscript{397}, a second similarly designed trial failed to detect any differences in a host of clinical and biochemical outcomes, notably including death, myocardial infarction, length of hospitalization, troponin-T, and CK-MB among patients that were prophylactically treated with N-acetylcysteine prior to CABG\textsuperscript{398}.

The second compound supplemented as an antioxidant during CABG is α-tocopherol, or vitamin E, which scavenges peroxyl radicals in biological lipid membranes\textsuperscript{399,400}. Similar to N-acetylcysteine, α-tocopherol appears capable of reducing markers of oxidative stress, but the overall clinical benefit appears to be limited\textsuperscript{401-403}. Interestingly, the intracoronary administration of α-tocopherol was found to improve post-reperfusion troponin-I and CK-MB profiles relative to control\textsuperscript{404}. These results suggest that both N-acetylcysteine and α-tocopherol must be applied intraoperatively to reduce ROS damage during ischemia-reperfusion injury, but the overall benefit in terms of clinical outcomes may be limited.

Finally, the intravenous anesthetic propofol (2,6-diisopropylphenol) has an intrinsic antioxidant capacity owing to its di-substituted phenolic structure. Like N-acetylcysteine, propofol is capable of scavenging \textsuperscript{•}OH\textsuperscript{405,406}, but not necessarily \textsuperscript{O}_2\textsuperscript{•}\textsuperscript{-}\textsuperscript{407}. Propofol completely inhibits \textit{in vitro} lipid peroxidation of a linoleic acid emulsion at a concentration beyond 140 µM, which, although exceeding clinically relevant concentrations by an order of magnitude, is more potent than α-tocopherol\textsuperscript{405}. Importantly, phenolic antioxidants can structurally incorporate a nitroso- group in the presence of NO\textsuperscript{-}, which decreases their antioxidant capacities\textsuperscript{408}. Nevertheless, the concentration range of 10 µM and below of propofol can inhibit \textit{in vitro} lipid peroxidation. 

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peroxidation of artificial membrane and whole blood by 50%\textsuperscript{406,409}. Clinically relevant infusion rates can achieve propofol concentrations that enhance erythrocyte antioxidant capacity of during CABG with CPB\textsuperscript{410,411}. Propofol has also been shown to scavenge ONOO\textsuperscript{-} in cultured endothelial cells and astrocytes, in a reaction that appears to occur to a greater degree than to tyrosine nitration\textsuperscript{412,413}.

Several studies have demonstrated that propofol anesthesia during CABG is associated with a decrease in lipid peroxidation\textsuperscript{414-417}. Despite these results, it remains unclear whether propofol or volatile anesthetics produce significantly different outcomes subsequent to cardiac surgery. On one hand, several aforementioned studies indicate that volatile anesthetic preconditioning is associated with improved functional and troponin I profiles compared to propofol\textsuperscript{373,375-377}. On the other hand, other aforementioned studies fail to detect significant differences in several patient outcomes including mortality and morbidity\textsuperscript{382,383,385}. It may ultimately remain difficult to assess clinical differences in the absence of standard volatile anesthetic preconditioning protocols and measures of achieved propofol concentrations in whole blood.

1.8. Conclusions and perspectives

Myocardial ischemia and reperfusion is a major source of cardiomyocyte injury within the context of a host of clinical pathologies. These pathologies include cardiac arrest, acute myocardial infarction, and low cardiac output syndrome after cardiac surgery. The financial and societal relevance of this type of injury is underscored by the increasing prevalence of heart disease. Any improvement in the clinical outcomes associated with these pathologies must be predicated on reducing the magnitude of
ischemia-reperfusion injury, which by extension requires an understanding of the central role that oxygen and nitrogen radicals play in its pathogenesis.

There are several potential sources of free radicals during tissue ischemia and reperfusion, but mitochondria appear to be especially important in the context of the myocardium. Several factors underscore this reasoning: 1. The constant energy demand of the myocardium dictates that cardiac myocytes contain large amounts of mitochondria. 2. Mitochondria are an important source of $O_2^*$, 'OH, and NO' generation during ischemia and reperfusion. 3. Mitochondria contain all basic types of biomolecules that would be susceptible to free radical reactions in the cell as a whole. Furthermore, the mitochondrial membranes contain high levels of unsaturated fatty acids that are susceptible to peroxidation reactions. 4. Mitochondria are key regulators of ROS mediated cell death. Although xanthine oxidase and inflammatory mediators likely contribute to the myocardial pool of $O_2^*$, especially in the diseased heart, and 'OH can mediate its toxicity throughout the cell, cardiac mitochondria likely represent the most important source and site of myocardial ROS generation during myocardial ischemia and reperfusion. As such, mitochondria serve as a useful microcosm of the cardiomyocytes in which they reside; their protection from ROS-mediated damage during ischemia and reperfusion may pay dividends beyond even their myocyte hosts.

1.9. Thesis objectives and outline

The unsaturated fatty acids that comprise cellular and subcellular lipid membranes are susceptible to ROS-mediated peroxidation reactions. These self-propagating reactions are initiated by 'OH, and yield a heterogeneous array of products that encompass acid-catalyzed fragmentation products including MDA, as well as a host of isoprostane...
lipoxygenases. Lipid peroxidation products can adversely affect the functional integrity of lipid membranes, and are therefore quickly cleaved and released by membrane phospholipases.

One isoprostane isomer, 15-F_{2\tau}-isoprostane, induces dose-dependent vasoconstriction in the coronary vasculature with sub-\mu M potency. Levels of 15-F_{2\tau}-isoprostane increase during coronary angioplasty and CABG procedures, it has been associated with a decline in postoperative cardiac function, and its formation may exacerbate or prolong cardiac dysfunction after ischemia and reperfusion. Intraoperative ischemia-reperfusion injury does not universally translate to persistent or significant decreases in postoperative clinical outcomes, so the ability to detect additional clinical outcome advantages is often limited to large, multi-centered, and adequately powered trials. By contrast, effective lipid soluble antioxidant therapies should reduce 15-F_{2\tau}-isoprostane generation in a patient cohort at large.

In light of previous results that point toward a propofol-mediated cardioprotective effect, and given the duality of 15-F_{2\tau}-isoprostane as an indicator of ROS-mediated lipid peroxidation and a mediator of myocardial ischemia-reperfusion injury, this thesis is set around the following central hypothesis:

**Propofol reduces the incidence of low cardiac output syndrome subsequent to CABG with CPB by decreasing the magnitude of 15-F_{2\tau}-isoprostane generation during myocardial ischemia and reperfusion.**

Implicit in this hypothesis is that propofol’s antioxidant capacity can decrease \cdot OH-mediated, self-propagated lipid peroxidation. Of equal importance, \cdot OH generation can be decreased by a reduction in ONOO\^{-} formation. This reduction in ONOO^{-} formation
can in turn result from a decrease in the generation of its reactants, namely NO• and O₂•-
. Accordingly, reducing NO• generation or temporally separating NO• and O₂•- generation
could influence the magnitude of ‘OH, and subsequently 15-F₂-isoprostane generation
during ischemia and reperfusion. Accordingly, this thesis aims to address the following
research questions:

1. Does propofol reduce the magnitude of ‘OH-initiated, self-propagating lipid
peroxidation reactions over isoflurane preconditioning as evidenced by 15-F₂-isoprostane generation during ischemia and reperfusion within the context of
CABG with CPB?

2. Is NO• generation during ischemia and reperfusion differentially affected by
propofol or isoflurane preconditioning during CABG with CPB?

3. Does propofol differ from isoflurane preconditioning in terms of ONOO•
generation, during myocardial ischemia and reperfusion injury as evidenced by
plasma 3-nitrotyrosine levels within the context of CABG with CPB?

The central hypothesis also postulates that the conditions that yield propofol-based
cardioprotection in laboratory models of ischemia-reperfusion injury translate to the
clinical setting, and that previous results indicative of the inability of isoflurane-
preconditioning to decrease ’OH generation are robust. Consequently, several clinical-
based cardioprotective strategies will briefly be reviewed in Chapter 2, followed by a
discussion of the pharmacology of propofol, its antioxidant capacity from the perspective
of relevant laboratory-based studies, and the precedents that exist in the literature in
support of its use as a potential cardioprotectant.
The flow chart in Figure 7 is a general outline of this thesis. The development of a clinical study capable of addressing the central hypothesis underscores the objectives of this thesis. This clinical study required a maneuver that could reliably and predictably deliver a concentration of propofol to the heart at reperfusion that corresponds to concentrations that tend to be associated with laboratory-based cardioprotection. Accordingly, Chapter 3 describes the development of a capillary electrophoresis-based technique that enables rapid and quantitative analysis of propofol in whole blood from patients undergoing CABG with CPB.

The analytical technique described in Chapter 3 was used within the context of a dose finding study. The results from this dose-finding study, which is described in Chapter 4, helped inform the clinical parameters that translate laboratory-based conditions for propofol-based cardioprotection to a clinical study. Chapter 5 describes the rationale and design of this larger clinical study, within which the research questions and central hypothesis can be addressed. Finally, preliminary results that address the research questions are presented in Chapter 6. This thesis concludes with a summary of findings, with future directions for exploration, and with a prospective outlook of the research.
Figure 7: Flow chart describing the conceptual link between the individual chapters of this thesis.
2. Clinical-based cardioprotection beyond volatile anesthetic preconditioning

Patients presenting for cardiac surgery are a heterogeneous clinical population. This heterogeneity may be reflected by one patient receiving a slight but significant outcome benefit from a given treatment or procedure, to the detriment or indifference of another. For example, less severe coronary artery disease can be treated pharmacologically or surgically without significant differences in outcomes\textsuperscript{4,5}, but medium- and higher-risk patients with more severe disease, and those with co-morbidities clearly benefit from reduced mortality when treated with CABG compared to non-surgical management\textsuperscript{8} and percutaneous interventions\textsuperscript{6,9,10}. A particularly significant and prevalent co-morbidity is diabetes mellitus.

Mortality from cardiovascular pathologies is doubled among patients with diabetes, and these patients, who are two to five times more likely to develop cardiovascular disease and have smaller vessel diameter, often have multi-vessel disease and lower left ventricular ejection fraction at a significantly greater frequency than their non-diabetic counterparts\textsuperscript{424}. Unsurprisingly, diabetics represent 30\% of patients presenting for cardiac surgery\textsuperscript{21}. Although patients with diabetes mellitus may benefit from CABG over percutaneous intervention\textsuperscript{6,7}, their condition is associated with an increased rate of early and late adverse postoperative events following cardiac surgery, including increased perioperative morbidity and mortality, reduced long term survival, and recurrence of angina\textsuperscript{12,13,16,425-429}.

In the early postoperative period, low cardiac output syndrome affects up to 26\% of diabetic patients, compared to 8\% to 15\% of non-diabetic patients recovering from cardiac surgery\textsuperscript{14-16}. This syndrome is defined by persistent hypotension (a systolic blood
pressure less than 90 mmHg) and/or low cardiac index (less than 2.2 L·min·m⁻²) despite hemodynamic optimization, and can quadruple the overall mortality rate for CABG surgery from 2% to 8%. Intraoperative ischemia-reperfusion injury is a major factor in the development of low cardiac output syndrome, so its disproportionate frequency among patients with diabetes reflects either their increased susceptibility to ischemia-reperfusion injury and/or their decreased susceptibility to intraoperative cardioprotection.

Isoflurane preconditioning mimics ischemic preconditioning with respect to \( \text{K}_{\text{ATP}} \) channel activation. Both isoflurane and ischemic preconditioning recruit Akt signaling and Bcl-2, but they appear to diverge in their ability to decrease \( \cdot \text{OH} \) generation at reperfusion – ischemic preconditioning being capable of doing so, but not isoflurane. This divergence may reflect the finding that isoflurane only marginally improves outcomes subsequent to CABG. Alternatively, Akt signaling and Bcl-2 activation may sufficiently induce a protective phenotype in a subset of patients so that the additional benefit of decreased \( \cdot \text{OH} \) generation is not necessary for cardioprotection.

Experimental models of diabetes suggest that signal transduction pathways required for ischemic or anesthetic preconditioning are corrupted. Hyperglycemia-induced oxidative stress can suppress myocardial \( \text{K}_{\text{ATP}} \) channel activity and inhibit Akt signaling. These differences are illustrated clinically by different gene expression profiles between the diabetic and non-diabetic myocardium subsequent to CPB. Concerning the ROS-induced end-effectors of ischemia-reperfusion injury, experimental studies indicate that mitochondrial permeability transition may be enhanced by diabetes, while MMP expression and activation is increased by
hyperlipidemia\textsuperscript{446} and diabetes\textsuperscript{447, 448}. These results underscore and reflect in the notion that volatile anesthetic preconditioning is compromised in the diabetic myocardium\textsuperscript{436, 449}, and that therapeutic approaches that demonstrated clinical effective cardioprotection in these patients remain largely elusive\textsuperscript{449}.

2.1. Propofol-mediated, antioxidant-based cardioprotection

A recent meta analysis of observational studies found that the integrity of endogenous antioxidant defenses inversely correlated with clinical outcomes in patients with coronary artery disease\textsuperscript{450}. These data suggest that patients with coronary artery disease have insufficient or depleted endogenous defenses to ROS, which would make them vulnerable to the ROS that are generated during myocardial ischemia-reperfusion injury. Antioxidants have the potential to benefit patients during cardiac surgery by intercepting and neutralizing ROS generated during ischemia and reperfusion. In doing so, antioxidants could inhibit ROS-activated apoptotic processes such as mitochondrial permeability transition, and decrease the activation of MMPs, while the integrity of cellular and sub-cellular membranes and membrane-protein complexes. Although this concept tends to bear fruit in experimental models of ischemia and reperfusion, clinical evidence is more ambiguous (reviewed in\textsuperscript{451}).

Effective antioxidant therapy represents an attractive opportunity because it is mechanismically orthogonal to isoflurane-based preconditioning and accordingly circumvents signaling pathways that may be compromised in the diabetic heart. Clinical studies of adjuvant antioxidant therapy indeed tend to show decreases in surrogate markers of oxidative stress, but benefits in terms of patient outcomes or markers of myocardial damage are less evident. One likely reason behind the marginal clinical
benefits is that the majority of patients who undergo cardiac surgery emerge without major intraoperative myocardial injury that translates into prolonged or significantly depressed clinical outcomes, so the detection of any additional clinical outcome advantages necessitates much larger studies of adequate power.

The contrasting results between laboratory models and clinical studies may also indicate that antioxidant therapies have not been effectively translated. Specifically, antioxidants are likely compartmentalized, eliminated, or otherwise diluted in vivo, so that their myocardial concentrations no longer reflect experimental ones. To this end, intracoronary administration of α-tocopherol was found to improve post-reperfusion troponin-I and CK-MB profiles relative to control and its intraoperative administration marginally improved myocardial function. Conversely, its prophylactic application has no bearing on markers of myocardial injury in the early postoperative period.

Similarly, N-acetylcysteine administration during CPB or as a cardioplegia solution supplement is capable of reducing myocardial oxidative stress, at times with decreased markers of myocardial injury, while prophylactic N-acetylcysteine administration again has no bearing on any marker of postoperative myocardial damage or functional outcome.

2.2. Propofol modulation of ion fluxes

Propofol is an intravenous anesthetic whose lipophilicity accounts for its rapid distribution half-life (2-8 minutes), and whose high rate of clearance, which exceeds hepatic blood flow, accounts for a relatively quick post-operative arousal time. The anesthetic activity of propofol ultimately stems from its ability induce membrane hyperpolarization. The mechanism by which this is accomplished is derived from
propofol’s ability to bind and modulate a host of receptors across a small concentration range, these include glycine receptors\textsuperscript{457}, voltage dependent Na\textsuperscript{+} channels\textsuperscript{458}, and chiefly GABA\textsubscript{A} receptors (reviewed in\textsuperscript{459}). Interestingly, propofol positively modulates GABA\textsubscript{A} activity in two separate and independent ways: first, propofol increases the open probability of the channel in the presence of GABA by binding the GABA\textsubscript{A} receptor independently of its subunit composition\textsuperscript{457,460}; second, propofol directly activates GABA\textsubscript{A} by binding near the extracellular site of a transmembrane motif on the β-subunit of this receptor\textsuperscript{460-465}.

The whole blood concentration of propofol at which half of patients are awake is 1.07 µg/mL, while the average surgical concentration varies from 2.97 to 4.05 µg/mL, depending on the procedure\textsuperscript{466}. Propofol induces significant effects on ionic gradients in the myocardium within this concentration range. Specifically, propofol directly reduces the open probability of L-type Ca\textsuperscript{2+} channels\textsuperscript{467,468} and decreases inward Ca\textsuperscript{2+} current starting at concentrations of 4.5 µg/mL\textsuperscript{469,470}. Interestingly, propofol appears to offset these actions by increasing myofilament sensitivity to calcium\textsuperscript{471,472}, thus minimizing any effect on cardiac contractility. Furthermore, its negative inotropic action is not apparent at clinically relevant concentrations\textsuperscript{472}.

Propofol causes a hyperpolarizing shift with decreased peak sodium current amplitudes, slowed rates of recovery from inactivation through reduced single Na\textsuperscript{+} channel open duration, and dose-dependently blocked whole cell Na\textsuperscript{+} currents in rat ventricular myocytes\textsuperscript{458}. Although this inward Na\textsuperscript{+} current-reducing effect has not directly been implicated in cardioprotection, it likely contributes to propofol’s overall ability to reduce intracellular Ca\textsuperscript{2+} concentrations, as would its ability to induce PKC-
dependent Na\(^+\)/Ca\(^{2+}\) exchanger reversal and its preservation of Na\(^+\)/H\(^+\) exchanger activity\(^{473-476}\).

2.3. **Propofol mediated reactive oxygen species scavenging**

Propofol is a phenol that is substituted at the 2- and 6- position with isopropyl groups. This structure resembles that of tocopherols (Figure 8), and is thought to contribute to propofol’s antioxidant activity, which mirrors that of \(\alpha\)-tocopherol\(^{405,477}\). Propofol’s capacity to scavenge \(\cdot\)OH radicals in vitro is generally acknowledged\(^{405,406}\), and although there is some question whether this capacity extends to \(O_2\cdot^-\)\(^{407}\), it clearly decreases MDA, a marker of lipid peroxidation\(^{405-408}\). This capacity is illustrated by the finding that 140 \(\mu\)M of propofol completely inhibits *in vitro* lipid peroxidation of a linoleic acid emulsion. Although this concentration exceeds clinical relevance by more than an order of magnitude, the effect was more potent than that of \(\alpha\)-tocopherol\(^405\). At the more relevant concentration range of 10 \(\mu\)M, propofol can inhibit *in vitro* lipid peroxidation of artificial membrane and in whole blood by 50\%\(^{406,409}\).

Experimentally, propofol dose-dependently reduces MDA production in red blood cells\(^{409,410,478}\), cultured cardiomyocytes\(^{479}\), and isolated heart models of ischemia-reperfusion injury\(^{480-483}\). These isolated heart studies, and similar ones that did not measure MDA levels\(^{484-486}\), report reductions in measures of ischemia-reperfusion injury with improved functional endpoints. One such study reported that propofol during ischemia and reperfusion can reduce mitochondrial lipid peroxidation and \(H_2O_2\) production in association with preserved mitochondrial respiration and cardiolipin content\(^{487}\). This study extrapolated that 5 \(\mu\)g/mL of propofol in patient blood would be expected to reduce the severity of myocardial ischemia-reperfusion injury.
2.4. Precedents for propofol-mediated cardioprotection

In 2005, Lim et al reported a swine model of cardioplegic arrest with CPB, which serves as a clinically relevant model of CABG with CPB. This study compared pigs receiving propofol during CPB with those receiving sodium thiopentone-diazepam-fentanyl intravenous anesthesia throughout. Pigs receiving propofol had reduced troponin I levels at reperfusion, reduced hemodynamic dysfunction after CPB, and reduced ischemia-reperfusion injury as evidenced by preserved myocardial tissue levels of adenine nucleotides, lactate, and amino acids. The estimated a propofol concentration in blood at 3.7 μg/mL.

Although Lim et al did not measure markers of lipid peroxidation, several other clinical studies do. Specifically, propofol’s ability to enhance erythrocyte antioxidant capacity appears to extend to CABG with CPB, provided sufficient drug concentrations...
are achieved in blood\textsuperscript{410,489}. Similarly, several studies have demonstrated that propofol anesthesia during CABG is associated with decreased lipid peroxidation\textsuperscript{414-417,490}. A critical difference between experimental models of myocardial ischemia-reperfusion injury and the clinical setting is that increased lipid peroxidation may in part reflect an inflammatory response to intraoperative tissue damage. To this end, propofol has been shown to decrease systemic inflammation and lipid peroxidation during and after aortic crossclamp application in pigs subject to myocardial ischemia-reperfusion injury\textsuperscript{491,492}. Likewise, propofol reduces iNOS activity and inflammation during general surgery\textsuperscript{493} and improves pulmonary inflammation subsequent to CPB in patients\textsuperscript{494}. Unfortunately these results can neither attribute the decreased lipid peroxidation to reduced ROS generation in the myocardium nor to a reduction in the inflammatory response. In a similar vein, one clinical study found that patients receiving an average of 7 µg/mL of propofol had decreased indices of post-ischemic inflammation and significantly lower levels of MDA in coronary sinus blood immediately after crossclamp removal, but no significant differences in post-operative MDA generation in systemic blood\textsuperscript{416}. A subsequent study from the same group reported similar findings with respect to MDA\textsuperscript{417}.

Another critical difference between experimental models of myocardial ischemia-reperfusion injury and its clinical manifestation is that volatile anesthetics likely influence myocardial tolerance to ischemia. As such, clinical studies, in which several choices for the primary anesthetic have demonstrated some laboratory based benefit, almost certainly lack a clear control group. In this light, it remains unsurprisingly unclear whether propofol can significantly improve outcomes over volatile anesthetics during cardiac surgery. On one hand, several studies indicate that volatile anesthetic
preconditioning is associated with improved functional and troponin I profiles compared to propofol\textsuperscript{373, 375-377}. On the other hand, other studies fail to detect significant differences in several patient outcomes including mortality and morbidity\textsuperscript{382, 383, 385}.

A meta-analysis comparing sevoflurane to propofol anesthesia found that patients receiving sevoflurane generally had higher post-bypass cardiac indices, decreased serum troponin I, shorter intensive care unit and hospital stays, and lower incidence of myocardial ischemia, but failed to detect any significant differences in postoperative ventilation time, inotropic support, mortality, myocardial infarction, and atrial fibrillation\textsuperscript{382}. These latter results are in agreement with another retrospective review comparing sevoflurane to propofol anesthesia in 10,535 patients undergoing cardiac surgery, which failed to detect significant differences in 30-day mortality, post-operative myocardial infarction and arrhythmia. This second study suggested that propofol and sevoflurane likely conferred mechanistically distinct cardioprotective effects\textsuperscript{383}. In a similar vein, a recent study comparing sevoflurane, isoflurane and propofol during CABG found that patients receiving volatile anesthesia had improved troponin I profiles while those receiving propofol had less lipid peroxidation, improved glutathione peroxidase activity, and decreased NO\textsuperscript{•} production\textsuperscript{384}. Finally, a more comprehensive study comparing isoflurane and propofol anesthesia failed to find any significant differences in postoperative troponin-I, hospital or intensive care unit stays, or in hospital-, 30-day- and 1-year cardiac morbidity and mortality\textsuperscript{385}. 
3. Quantitative analysis of propofol in whole blood using capillary electrophoresis

3.1. Introduction

Propofol is a frequently used intravenous anesthetic for the introduction and maintenance of anesthesia. Our research group is interested in the potential cardioprotective effects of propofol during CPB. Target controlled infusion devices, which predict whole blood propofol concentrations based on mathematical algorithms that link patient characteristics and pharmacokinetics with infusion rates, are currently in use. Unfortunately, there are often discrepancies between predicted and achieved concentrations. Furthermore, target controlled infusion devices are not universally approved for clinical use. In order to overcome these limitations, techniques and devices capable of determining actual drug concentrations in whole blood are required. In order to maximize their clinical usefulness, analytical devices should be simple, highly automated, fast, accurate, and precise.

Several methods for the quantitative determination of propofol in biological samples have been reported, examples include high performance liquid chromatography-UV spectrophotometry\textsuperscript{495-498}, gas chromatography with flame ionization detection\textsuperscript{498, 499}, and chromatography techniques coupled with mass spectrometry detectors\textsuperscript{497-502}. While these techniques offer accurate and precise quantitative results, they have qualities that do not necessarily translate well to the clinical setting. Specifically, mass spectrometry techniques that measure propofol concentrations in expired breath are rendered void by the use of CPB. Mass spectrometry detectors usually require front-end separation techniques when analyzing biological samples that originate from complex matrices such
as blood. Liquid chromatography-based techniques can produce efficient and fast separations, but they often require longer column regeneration steps between runs that involve larger organic solvent volumes. A final impediment to the routine use of many modern separation techniques for target-achieved type analysis in the clinical context is the physical size of commercially available mass spectrometry, liquid chromatography, and capillary electrophoresis (CE) instruments.

CE is one of the most powerful tools for chemical separation. The advantages of high resolution, short analysis time, low cost, and small buffer and solvent volume requirements make CE an attractive technique to analyze complex matrices\(^{503, 504}\). Although the size of commercially available CE instruments precludes their routine presence in a clinical setting, the much smaller microfluidic devices have the potential to meet this need. The conceptual similarities between CE and electrophoretic-based microfluidic separations represents a strong opportunity to foster the development of target-achieved drug delivery strategies and devices, in which drug dosing is dictated by actual concentrations achieved in whole blood, that are better suited to routine use in clinical settings.

The aim of the present study was to develop and validate a CE-based method capable of quantitative propofol analysis in whole blood. Propofol has an estimated theoretical triglyceride-water partition coefficient of \(\lambda_{tr/w} = 4715\)\(^{505}\), it strongly binds to plasma proteins and cellular blood constituents, and it has a mean free fraction in plasma of 1-3%\(^{506}\). Capillary electrophoresis with micellar additives, also called micellar electrokinetic chromatography (MEKC), first introduced by Terabe et al.\(^{507}\), has been widely used for the analysis of biological samples\(^{508}\), particularly where neutral and
hydrophobic compounds are concerned. We have developed and validated a fast and highly selective MEKC-based method for the quantitative analysis of propofol in whole blood using a commercially available CE system. This method was used to determine the concentration of propofol in whole blood samples obtained from patients undergoing CABG with CPB.

3.2. Experimental

3.2.1 Apparatus

All experiments were carried out on a Beckman Coulter P/ACE MDQ System (Beckman Coulter Inc., Fullerton, CA, USA) with a UV absorption detector. The detection wavelength that was used for the analysis of propofol was 200 nm. An uncoated fused-silica capillary (50 cm total length, 40 cm length to detector, 50 µm inner diameter, 360 µm outer diameter) (Polymicro Technologies, Phoenix, AZ, USA) was used throughout. A separation temperature of 25°C was maintained for all CE experiments.

3.2.2 Chemicals and reagents

Borax and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Oakville, ON, Canada). Separation buffer consisted of an aqueous solution containing 50 mM sodium dodecyl sulfate and 15 mM borax. This separation buffer was sonicated and filtered through a 0.45 µm membrane filter. Tetramethylammonium hydroxide (25% in methanol) was purchased from Alfa Aesar (Ward Hill, MA, USA) and diluted before use with HPLC grade 2-propanol (Fisher Scientific, Ottawa, ON, Canada) (3:37 v/v). Cyclohexane, acetonitrile, and methanol were purchased from Fisher Chemicals (Fisher Scientific, Ottawa, ON, Canada) Deionized water was obtained using a NANOpure
Infinity Reagent Grade Water System (Apple Scientific Inc., Chesterland, OH, USA). All solutions were filtered through 0.45 µm membrane filters (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) prior to use. Propofol (2,6-diisopropylphenol), 97% purity, was purchased from Sigma-Aldrich. Thymol, 99% purity, was purchased from Acros Organics (Morris Plains, NJ, USA) and used as internal standard. Durapore centrifugal filters with 0.1 µm pores for the liquid-liquid extracted solutions, were purchased from Millipore, Inc. (Bedford, MA, USA).

3.2.3 Preparation of standard solutions

Initial stock solutions of propofol and thymol were prepared separately in 50% acetonitrile-50% deionized water. These solutions consisted of 800 µg/mL propofol and 100 µg/mL of thymol. Propofol was subsequently serially diluted with 6% acetonitrile to the following desired concentrations: 16, 8, 4, 2, 1, 0.5 µg/mL. Two additional concentrations (0.3, 0.1 µg/mL) were prepared from 1 µg/mL. These solutions were supplemented with thymol stock solution for a final internal standard concentration of 7 µg/mL.

3.2.4 Patients and sampling

This investigation conforms to the principles outlined in the Declaration of Helsinki. Following institutional approval and written informed patient consent, 30 patients scheduled for primary CABG surgery were enrolled in an ongoing parallel study investigating the short-term application of propofol during CPB. All patients received a 1.0 mg/kg bolus dose of propofol at heparinization (approximately 10 minutes prior to aortic crossclamp placement) followed by an infusion of 120 µg•kg⁻¹•min⁻¹ for the
duration of CPB. A whole blood sample of 5 mL was withdrawn from the central venous line 15 minutes after aortic declamping using a vacutainer containing heparin (Becton Dickinson, NJ), and stored in 1.25 mL aliquots at -80°C for subsequent CE analysis. Patient and operative characteristics are listed in Table 1. Blank venous blood, which did not contain propofol, was sampled from the central venous line intraoperatively in the interim time between induction of anesthesia and the administration of propofol. This blood was sampled and stored as described for central venous samples. Perioperative patient care was administered according to the routine clinical practice at Vancouver General Hospital.

**Table 1:** General patient and operative characteristics.

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</table>
3.2.5 Sample extraction

A liquid-liquid extraction procedure described by Plummer\(^{509}\) is frequently used for the analysis of propofol in blood samples. We have modified this procedure to suit the CE analysis. In brief, 30 µL of thymol internal standard stock solution (100 µg/mL), 200 µL of deionized water, and 25 µL of 1 M NaH\(_2\)PO\(_4\) (pH 4.2) were added to a 400 µL aliquot of thawed whole blood. The sample mixture was then vortexed for 1 min, after which 650 µL of cyclohexane was added, followed by at least 2 minutes of inverting and vortexing. Organic and aqueous layers were separated by centrifugation (1200 rcf for 1 minute at 4°C). A 500 µL aliquot of the cyclohexane layer was transferred through a 0.1 µm centrifugal filter into a clean tube containing 10 µL of diluted Tetramethylammonium hydroxide solution (2% v/v in propanol). The solvent was evaporated to dryness under vacuum centrifuge (down to 0.6 torr) and the residue was re-dissolved in 80 µL of 6% acetonitrile in water.

3.2.6 Capillary electrophoresis

Prior to daily use, the capillary was conditioned with 0.1 M NaOH, methanol, purified water, and separation buffer in successive rinses of 20 minutes each. Individual CE runs were preceded by four successive 3 minute rinses using 0.1 M NaOH, methanol, purified water, and separation buffer. A pressure difference of 20 psi was used in all rinse procedures.

Upon filling the capillary with separation buffer, conventional hydrodynamic sample injection was performed at 0.5 psi (3447 Pa) for 5 seconds. A separation voltage of 25 kV under normal polarity was applied continuously for 12 minutes. Propofol and thymol were detected with UV absorbance at λ=200nm.
3.3. Results and discussion

Propofol is a highly lipophilic compound\textsuperscript{505} with a pKa of 11. We developed a MEKC-based separation to overcome the low aqueous solubility of the drug in its neutral form in solutions with pH < 11. Specific aspects of the method development process for the quantitative analysis of propofol in whole blood are described below.

3.3.1 Specificity

In order to identify the thymol and propofol peaks, and to match them with specific migration times and apparent electrophoretic mobilities, we added either propofol or thymol reference materials into the blank- and blank whole blood matrices. The peak identities were further confirmed during the construction of the calibration curve, in which a series of standard solutions were prepared with increasing propofol concentrations but one consistent thymol concentration. Accordingly, the propofol peak could be distinguished from the thymol peak because the former had an incrementally increasing peak area.

In CE analysis, apparent electrophoretic mobility ($\mu_{ep}^{A}$) is more representative of a given analyte than migration time because it normalizes the rate of migration with respect to both the medium and the electric field strength, and is independent of electroosmotic flow. Accordingly, our group always converts migration times to electrophoretic mobility when conducting CE. The apparent electrophoretic mobilities of thymol and propofol always have coefficients of variation below 0.7 (Table 2, Table 3, Table 5).

Based on specific apparent electrophoretic mobility, we can assign thymol and propofol peaks in different samples without ambiguity. In a similar vein, there appears to
be a difference between the migration times of propofol and thymol from Figure 9a to Figure 9b. These electropherograms were produced with two separately prepared capillaries. This difference may manifest itself in the electroosmotic flow, and therefore alter the migration times. Calculating apparent electrophoretic mobilities reveals the magnitude of the actual difference (Figure 9a: $\mu_{ep}^T = -22.08 \text{ cm}^2 \cdot \text{kV}^{-1} \cdot \text{min}^{-1}$, $\mu_{ep}^P = -23.08 \text{ cm}^2 \cdot \text{kV}^{-1} \cdot \text{min}^{-1}$; Figure 9b: $\mu_{ep}^T = -22.19 \text{ cm}^2 \cdot \text{kV}^{-1} \cdot \text{min}^{-1}$, $\mu_{ep}^P = -23.22 \text{ cm}^2 \cdot \text{kV}^{-1} \cdot \text{min}^{-1}$).

**Table 2:** Reproducibility of three clinically relevant standard propofol concentrations on the same day.

<table>
<thead>
<tr>
<th></th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>$\mu$</th>
<th>$\sigma$</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area corr</td>
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<td>0.138</td>
<td>0.148</td>
<td>0.145</td>
<td>0.006</td>
<td>4.3</td>
</tr>
<tr>
<td>$\mu_{ep}^A$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T$</td>
<td>-21.99</td>
<td>-22.07</td>
<td>-22.10</td>
<td>-22.05</td>
<td>0.06</td>
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<tr>
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<td>-23.11</td>
<td>-23.07</td>
<td>0.06</td>
<td>0.3</td>
</tr>
<tr>
<td>$t_{mig}$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T$</td>
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<td>6.72</td>
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<td>$P$</td>
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<td>7.34</td>
<td>7.36</td>
<td>7.34</td>
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</tr>
<tr>
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<td>-21.90</td>
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<td>$t_{mig}$</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$T$</td>
<td>6.77</td>
<td>6.89</td>
<td>6.93</td>
<td>6.86</td>
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<tr>
<td>$P$</td>
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<td>7.58</td>
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<tr>
<td>$T$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>6.90</td>
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<td>7.56</td>
<td>0.14</td>
<td>1.9</td>
</tr>
</tbody>
</table>

P= Propofol; T= Thymol; Area corr= corrected area; $\mu_{ep}^A$= apparent electrophoretic mobility (cm$^2$·kV$^{-1}$·min$^{-1}$); $t_{mig}$= migration time (min); $\mu$ = mean; $\sigma$ = standard deviation; % RSD = relative standard deviation expressed as a percentage.
Table 3: Reproducibility of three clinically relevant standard propofol concentrations across three days.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Area$_{corr}$ P/T</th>
<th>$\mu$$_{ep}$ A</th>
<th>$\sigma$</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µg/mL</td>
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<td>-22.01</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-23.07 P</td>
<td>0.16</td>
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<tr>
<td></td>
<td></td>
<td>6.72 T</td>
<td>6.68</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.34 P</td>
<td>0.07</td>
<td>0.9</td>
</tr>
<tr>
<td>4 µg/mL</td>
<td></td>
<td>-21.90 T</td>
<td>-21.96</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-22.90 P</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.86 T</td>
<td>6.73</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.50 P</td>
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</tr>
<tr>
<td>8 µg/mL</td>
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<td>-21.95 T</td>
<td>-21.99</td>
<td>0.4</td>
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<td></td>
<td></td>
<td>6.90 T</td>
<td>6.76</td>
<td>2.2</td>
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<td></td>
<td></td>
<td>7.56 P</td>
<td>0.15</td>
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</tr>
</tbody>
</table>

P= Propofol; T= Thymol; Area$_{corr}$= corrected area; $\mu$$_{ep}$ A= apparent electrophoretic mobility (cm$^2$·kV$^{-1}$·min$^{-1}$); $t_{mig}$= migration time (min); $\mu$ = mean; $\sigma$ = standard deviation; % RSD = relative standard deviation expressed as a percentage.

The specificity of the method was also assessed by its ability to separate propofol and thymol from other nonspecific blood components, and to resolve the peaks from one another. In brief, the optimized method produced sharp, gaussian peaks for both propofol and thymol with baseline resolution ($R_s \geq 2.6$) at concentrations of 2, 4, and 8 µg/mL in both standard and sample extracted from whole blood matrices. Additionally, blank blood samples did not produce any detectable signals that interfered with the propofol and thymol peaks. Figure 9 shows the representative electropherograms for propofol and thymol from a) 6% acetonitrile and b) whole blood matrices.
Figure 9: Representative electropherograms of a) standard solution of propofol and thymol, and b) whole blood containing propofol and thymol. Run Conditions: Sample injection: 0.5 psi for 5 seconds. Run Buffer: 50 mM sodium dodecyl sulfate, 15 mM borax. Separation: 25 kV normal polarity over 12 minutes across an uncoated capillary (ID=50 μm; L_T=50 cm; L_D=40 cm). Detection: UV absorbance at λ=200 nm.
3.3.2 Selection of buffer type and separation voltage

The solubility of propofol is proportional to the concentrations of sodium dodecyl sulfate in the buffer, particularly because propofol exists almost entirely in its protonated and neutral in this buffer (pH=8.5). This limitation could be overcome by the addition of organic solvent in the running buffer, but this significantly compromised the consistency of run-times. The varying migration times reflect the difficulty of accurately controlling and reproducing the organic content in the buffer. The increased ionic strength of higher borax concentrations in the separation buffer translated into longer migration times. Alternatively, a lower ionic strength separation buffer resulted in broader peaks, lower resolution, and an inferior limit of detection. The composition of the separation buffer represents a compromise between the solubility and stacking efficiency of the analyte, and the running time. A 15 mM borax buffer (60 mM borate) with 50 mM sodium dodecyl sulfate was chosen as optimum condition based on the solubility of propofol, as well as the reproducibility and length of the runs.

The separation voltage of 25 kV across a 50 cm capillary of 50 µm inner diameter was chosen based on the highest potential difference within the linear region in the Ohm’s law plot (Figure 10). The optimized separation conditions produce propofol and thymol peaks in less than 8 minutes (thymol $t_{mig}$: 6.72±0.02 min; $\mu_{ep}^T$: -22.05±0.06 cm$^2$·kV$^{-1}$·min$^{-1}$; propofol $t_{mig}$: 7.34±0.02 min; $\mu_{ep}^P$: -23.07±0.06 cm$^2$·kV$^{-1}$·min$^{-1}$. Values in mean ± standard deviation).
Figure 10: Ohm's law plot generated to determine the optimal separation potential. (Capillary: $L_T = 50$ cm, 50 µm inner diameter, Buffer: 15 mM borax buffer with 50 mM sodium dodecyl sulfate).

3.3.3 Linearity

The clinically relevant concentration range of propofol was estimated between 1.5 and 10 µg/mL\textsubscript{(whole blood)}\textsuperscript{410,454}. The linearity of the current method was assessed using the corrected area of standard propofol solutions between 0.1 and 16 µg/mL relative to that of a fixed concentration of 7 µg/mL thymol. This ratio was plotted against the propofol concentration. This curve has a goodness of fit of $r^2 = 0.9995$ throughout the tested range, with the equation: $y = 0.0740x + 0.0019$ (slope: 0.00064, 95% CI slope: 0.0725 to 0.0760; y-intercept: 0.0039, 95% CI: -0.0075 to 0.0112, n=15 concentrations).
3.3.4 Reproducibility

Reproducibility was assessed by injecting 3 clinically relevant concentrations of propofol (2, 4, and 8 µg/mL) dissolved in 6% acetonitrile, and injected 3 times on the same day and on 3 successive days. The electrophoretic mobilities of propofol and thymol were calculated to assess the precision, selectivity, and specificity of the separation, while the corrected area ratio of the propofol and thymol peaks was used to measure the quantitative precision.

Table 2 and Table 3 summarize the results of the reproducibility assays for propofol at 2, 4 and 8 µg/ml on the same day and on 3 successive days, respectively. The apparent electrophoretic mobilities of thymol and propofol had acceptable consistency within a given day and throughout a three-day interval for each of the tested concentrations (% RSD ≤ 0.7). The consistency of the corrected area ratio was also acceptable on the same day and over the three days across the three tested concentrations (% RSD ≤ 4.3).

3.3.5 Optimization of the liquid-liquid sample extraction

The procedure used to extract propofol from whole blood was adapted from the liquid-liquid extraction first described by Plummer. We have modified this method to suit the requirement for CE analysis. The first modification was to run the liquid-liquid extracted organic layer through a syringe filter of 0.1 µm pore diameter in order to prevent the capillary from becoming plugged and to reduce the variation in the migration time. Additionally, rather than using a stream of N₂(g) to dry the extracted organic layer, we were able to achieve better precision using a vacuum centrifuge at 0.6 torr. We found
that the dry pellet could be stably stored under N\textsubscript{2}(g) at -80°C until subsequent resuspension and analysis with minimal degradation (data not shown).

In order to control the volatility and reduce the loss of propofol during the drying process, tetramethylammonium hydroxide needs to be added to the organic layer prior to drying and resuspension. In order to optimize the tetramethylammonium hydroxide content for subsequent CE analysis, we added 5, 15, 25, 35, and 45 µL of 1% tetramethylammonium hydroxide (in methanol) to the organic layer prior to drying and resuspension. The best electropherograms, in terms of resolution, peak height, and baseline stability, were obtained with 15 or 25 µL of 1% tetramethylammonium hydroxide. We subsequently made all of our tetramethylammonium hydroxide dilutions at 2% v/v in propanol because of its superior solubility with cyclohexane compared to methanol. Indeed, we found this change to yield better run-to-run reproducibility. The pH of the resulting resuspended sample solutions was consistently between 11 and 12.

We chose to use 400 µL blood and 650 µL cyclohexane to constitute the extraction system which satisfied the need for both accuracy and operability. 500 µL of organic phase out of 650 µL was then aspirated to preclude aqueous contamination. The smaller total volume of this extraction system (<1.5 mL) than that of the original design is not only more convenient from a clinical perspective, but also in the laboratory because common and disposable lab supplies and equipment such as eppendorf tubes, spin filters and microcentrifuges can be used. The precision and accuracy of the separation and recovery requires that errors in all upstream steps be minimized. Pipetting errors, weighing errors, and inconsistencies in the extraction steps tend to propagate throughout subsequent steps. This is especially true when smaller starting volumes of blood are used,
as with our method, because the relative error is larger. Weighing small quantities of solid should be avoided because they tend to yield relatively large sampling errors.

### 3.3.6 Optimization of the sample resuspension solution

We investigated the effects of the organic content and the ionic strength in the resuspension solution. Pellets were resuspended in aqueous solutions containing 6%, 30%, or 60% acetonitrile. 6% acetonitrile was a practical organic content from an analytical perspective because it was capable of keeping the analyte homogeneously dissolved without negatively affecting the peak shape. Higher organic contents tended to produce poor peak shapes. In order to optimize the ionic strength of the resuspension solution, we resuspended pellets in three buffers of increasing ionic strength. Solutions containing 6% acetonitrile with 0%, 1%, or 10% running buffer were studied. We found that the lowest ionic strength resuspension solution (6% acetonitrile with 0% running buffer) yielded the best resolution and peak shapes. We found that the main determinant for the pH of the extracted and prepared sample ready to be injected into the capillary was the amount of tetramethylammonium hydroxide that was added to the organic phase prior to drying under vacuum centrifuge, as opposed to the amount of borax buffer contained within the resuspension solution. These modifications enabled us to quantitatively analyze propofol from a 400 µL starting volume of whole blood.

### 3.3.7 On-line pH-difference induced focusing

The resuspended solutions consistently had a pH of between 11 and 12, which is similar to the pKa of both propofol (pKa = 11) and thymol (pKa = 10.5). Accordingly, propofol and thymol are likely fractionally ionized in the injected sample, establishing
equilibria between their respective anionic and neutral species. The analytes in the sample plug could experience an effect similar to pH junction velocity-difference induced focusing\textsuperscript{510}. In order to verify this effect we separated propofol and thymol in two distinct sample solutions: the first sample consisted of propofol and thymol resuspended in an aqueous solution containing 6% acetonitrile adjusted to a final pH of 12 with tetramethylammonium hydroxide. The second sample consisted of an equal concentration of propofol and thymol as condition 1, resuspended in 6% acetonitrile aqueous solution, with a final pH of 6.

Table 4 describes the peak width, migration time, velocity, and end length of the propofol and thymol peaks derived from these samples, as well as the resolution between them. These results, which show improved peak width, length, and resolution, suggest that our method induces an online focusing effect. We were able to achieve sufficient sensitivity for routine clinically relevant propofol concentrations, but this focusing effect could be further investigated to increase the sensitivity if needed.

**Table 4:** Contribution of sample solution pH to the resolution and focusing effect of the separation.

<table>
<thead>
<tr>
<th></th>
<th>Sample Solution pH 12</th>
<th>Sample Solution pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymol</td>
<td>Propofol</td>
</tr>
<tr>
<td>Peak Width (min)</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>Migration Time (min)</td>
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<td>7.30</td>
</tr>
<tr>
<td>Analyte Velocity (mm/min)</td>
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<td>54.79</td>
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<tr>
<td>Peak Length (mm)</td>
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<td>6.58</td>
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<td></td>
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</table>
3.3.8 Indices of precision and recovery of whole blood analysis

In order to assess the precision of the propofol analysis from whole blood, we spiked propofol into blank whole blood to obtain one of three clinically relevant final concentrations (2, 4, and 8 µg/mL). Thymol was subsequently added to a final concentration of 7 µg/mL. We performed the extraction, and analyzed the sample using our optimized CE method. Each concentration was independently prepared and analyzed in triplicate. The percent-recovery was calculated using the standard curve to convert the propofol/thymol (P/T) corrected area ratio of each sample to its corresponding propofol concentration, and expressing this value as a percentage of the actual spiked propofol concentration (equation 18).

Equation 18: \[ R = \frac{(P/T - b_{std, curve})m_{std, curve}}{[P_{spike}]} \times 100 \]

\( P/T \) denotes the corrected area ratio of propofol to thymol; \( b_{std, curve} \) and \( m_{std, curve} \) denote the y-intercept and slope of the standard curve; and \( [P_{spike}] \) denotes the concentration of propofol supplemented into the blank blood sample.

Table 5 shows indices of precision for propofol at 2, 4 or 8 µg/mL. The electrophoretic mobilities of propofol and thymol were consistent for each of the tested propofol concentrations (% RSD ≤ 0.2). The migration times of extracted samples were similar to those achieved using standard prepared solutions containing propofol and thymol, suggesting that the extraction and residual whole blood components did not significantly alter the separation process. Migration times of propofol and thymol peaks derived from extracted samples all have % RSD ≤ 0.1, indicating that the sample
preparation step consistently produces samples of similar composition that do not negatively influence the regeneration of the capillary after each separation process.

The consistency of the P/T corrected area ratio and the percent recovery reflect the cumulative error of the operator, the extraction, and the instrument. The coefficients of variation associated with these values are therefore expected to be elevated. Accordingly, the P/T corrected area ratio derived from propofol in whole blood has % RSD values at or below 5.2%, while the percentage recovery has % RSD values at or below 5.1%.

We report percent recovery values that consistently exceed 100% over the concentration range (2, 4, or 8 µg/mL) we examined (120.3%, 123.8% or 120.8%). The distribution and partition coefficients (log D at pH of 3-7, and log P, at 25°C) were calculated using Advanced Chemistry Development software (V8.14 for solaris ACD/Labs). Thymol has log D and log P values of 3.28, while those of propofol are 4.16. Accordingly, the efficiency with which propofol is extracted into cyclohexane is greater than that of thymol, and the P/T signal ratio subsequent to the extraction is therefore elevated. Since this elevated P/T signal ratio is used to derive the numerator in equation 18, the percent recovery can be expected to exceed 100%.
Table 5: Indices of precision of three independently prepared clinically relevant propofol concentrations spiked into and extracted from whole blood.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Area corr</th>
<th>P/T</th>
<th>% R</th>
<th>% RSD</th>
<th>µ</th>
<th>σ</th>
<th>µep</th>
<th>T</th>
<th>P</th>
<th>tmig</th>
<th>P/T</th>
<th>% R</th>
<th>% RSD</th>
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<tr>
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<td>0.181</td>
<td>0.166</td>
<td>0.176</td>
<td>0.009</td>
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<td>113.2</td>
<td>120.3</td>
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</tr>
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<td>4</td>
<td>0.359</td>
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<td>0.382</td>
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<td>0.015</td>
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<td>120.4</td>
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<td>4.9</td>
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<td>117.0</td>
<td>120.8</td>
<td>3.7</td>
<td>3.0</td>
<td></td>
</tr>
</tbody>
</table>

P= Propofol; T= Thymol; Area corr= corrected area; %R= % recovery; µep = apparent electrophoretic mobility (cm²·kV⁻¹·min⁻¹); tmig = migration time (min); µ= mean; σ= standard deviation % RSD = relative standard deviation expressed as a percentage. Prep= sample preparation

3.3.9 Limit of detection and quantitation

The limit of detection of a standard solution of propofol using our method was determined using the signal-to-noise ratio of 3 relative to the signal of the standardized thymol concentration (7 µg/mL). The slope from the standard curve was used to translate this P/T ratio to the corresponding propofol concentration. The limit of quantitation was determined similarly, using a signal-to-noise ratio of 10. The current method has a limit
of detection of 0.07 µg/mL and a limit of quantitation of 0.24 µg/mL. The above values are determined using thymol prepared in a neat solution, and therefore describe the limits of detection and quantitation for the instrument.

A second series of calculations were done using electropherograms derived from whole blood samples. The signal-to-noise ratio from these electropherograms includes the contribution of the extraction and the original whole blood matrix, and therefore more closely reflects the conditions for which the method was intended. The limit of detection and limit of quantitation values were determined as described above, but included a correction for the recovery (see section 3.8). The resulting limit of detection and limit of quantitation values from electropherograms of whole blood samples were 0.07 µg/mL and 0.23 µg/mL, respectively. The similarity between the noise from neat and extracted injections testifies to the effectiveness of the modified sample extraction procedure. Although these values are higher than what has been achieved with some other methods, we found that the sensitivity is sufficient for clinically relevant propofol concentrations with acceptable % RSD.

3.3.10 Patient samples

We used our method to analyze the concentration of propofol in the blood of 30 patients receiving propofol as the primary anesthetic during CPB. Whole blood samples were drawn 15 minutes after aortic declamping. Figure 11 shows the concentration distribution of these 30 patients. The average concentration of propofol was 5.36 µg/mL (95% CI 4.48 to 6.24 µg/mL). This concentration of propofol reflects the infusion rate of 120 µg•kg⁻¹•min⁻¹. The distribution in Figure 11 shows that propofol infusions normalized to patient weight results in a considerable amount of variation in the
concentration achieved in whole blood. The magnitude of this distribution is in agreement with previous reports\textsuperscript{454, 456, 502} and likely reflects both population variance and altered pharmacokinetics during CPB. Whatever the reason, the data show the need for a reliable method to monitor the actual whole blood concentrations achieved in patients during surgery. Such techniques will improve patient safety by reducing the likelihood of complications related to elevated concentrations, including the rare but fatal “propofol infusion syndrome”\textsuperscript{511}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{Concentration distribution of propofol in whole blood of 30 patients undergoing CABG with CPB. Samples were obtained 15 minutes after aortic declamping under a propofol infusion rate of 120 µg•kg\textsuperscript{-1}•min\textsuperscript{-1}. Run Conditions: Sample injection: 0.5 psi for 5 seconds. Run Buffer: 50 mM sodium dodecyl sulphate, 15 mM borax. Separation: 25kV normal polarity over 12 minutes across an uncoated capillary (ID=50 µm; LT=50 cm; LD=40 cm). Detection: UV absorbance at \lambda=200 nm.}
\end{figure}
3.4. Conclusion

We describe a MEKC-based method capable of quantitative propofol analysis in whole blood over a clinically relevant concentration range with acceptable precision. This method is capable of producing sharp, baseline-resolved analyte peaks in less than 8 minutes from 400 µL of whole blood. More importantly, the method is robust and accurate enough to provide reliable propofol concentrations from patient samples collected in clinic settings. The procedures described in this work that were aimed at improving the reproducibility and robustness should be applicable to method development for other pharmaceutical analysis. Target achieved type techniques will require analytical methods with a high degree of automation, fast run times, and small and simple instrumentation. We recognize that the current method does not meet all of these criteria, especially insofar as the sample preparation step is concerned. However, this accurate and reliable method will provide a foundation for the development of other types or devices that more closely meets this clinical demand.
4. Target achieved propofol concentration during on-pump cardiac surgery: A pilot dose finding study

4.1. Introduction

Ischemia-reperfusion injury during CABG is a source of intraoperative cardiac injury\textsuperscript{512}. Therapeutic pharmacologic options during surgery to preserve the viability of ischemic myocardium include volatile anesthetic pre- and post-conditioning and antioxidant therapies\textsuperscript{376, 513-515}. Unfortunately, the clinical reproducibility and effectiveness of volatile anesthetic preconditioning has recently come into question\textsuperscript{378, 380}. Anesthetic preconditioning has not translated easily to the clinical scenario and is not universally effective. Patient factors including diabetic status\textsuperscript{436, 449} and aortic crossclamp intervals exceeding 30 to 40 minutes\textsuperscript{516} could mitigate the effects of the preconditioning stimulus. Research into alternative approaches of cardioprotection is required.

Conditions at reperfusion significantly contribute to tissue injury and repair\textsuperscript{517}. The antioxidant\textsuperscript{409} and cell signaling properties\textsuperscript{518, 519} of propofol, as well as its ability to inhibit mitochondrial permeability transition\textsuperscript{420, 521} are well suited to reduce reperfusion injury. Unfortunately, clinical anesthetic conditioning studies have demonstrated that target controlled infusion devices, which predict whole blood propofol concentrations based on mathematical algorithms that link patient characteristics and pharmacokinetics with infusion rates, set to a target propofol concentration from 1 to 4 µg/mL failed to protect against myocardial injury\textsuperscript{375, 376}. Based on work from our lab and others using both simulated models of ischemia-reperfusion injury and studies in patients\textsuperscript{481, 482, 484, 485,
we postulate that propofol confers cardioprotection when a target range of 4.5 µg/ml to 8.9 µg/ml (25 to 50 µM) is achieved.

There have been limited in vivo studies evaluating the effect of increased propofol dosing to achieve the therapeutic concentration range defined in vitro. Recently, a clinically relevant swine model of normothermic blood cardioplegic arrest with CPB demonstrated that a 1 mg/kg bolus followed by a 100 µg•kg⁻¹•min⁻¹ continuous propofol infusion was cardioprotective without negative hemodynamic consequences⁴⁸⁸. The authors estimated whole blood propofol concentration of 3.7 µg/mL based on other clinical studies with similar operative procedures⁴⁵⁶, ⁵²⁵, ⁵²⁶. We previously found that a 2 to 2.5 mg/kg bolus of propofol followed by an infusion of 200 µg•kg⁻¹•min⁻¹ produces drug concentrations associated with increased antioxidant capacity (8.2+/− 2.1 µg/mL), but showed signs of intraoperative cardiac depression compared to conventional propofol or isoflurane anesthesia maintenance⁴¹⁰. More recently, increasing propofol anesthetic maintenance from 60 to 120 µg•kg⁻¹•min⁻¹ intraoperatively was associated with a reduction in biomarkers of cardiac injury and oxidative stress, although the range of values was consistent with those expected during cardiac surgery⁴¹⁴. Clinically relevant differences in hemodynamics and left ventricular function were not detected in this study and drug concentrations were not measured. Further systematic investigations are needed to evaluate the role of propofol in cardiac surgery.

It is unknown if experimental cardioprotective propofol concentrations can routinely be achieved at reperfusion during CABG with CPB using short-term continuous infusion, or if such concentrations are associated with an increased risk of cardiac instability upon
emergence from CPB. To address this question, we conducted a pilot dose finding study and developed a predictive mathematical modeling for optimal dosing in patients.

In this pilot study, we hypothesize that a whole blood propofol concentration of 5 µg/mL can be achieved clinically with continuous drug delivery during CABG with CPB. We focused our treatment interval to the CPB interval of CABG, and measured the resulting propofol concentrations in whole blood 15 minutes after reperfusion. We also sought to identify any evidence of clinically significant cardiac depression upon separation from bypass, and measured intraoperative hemodynamic performance using cardiac index, systemic vascular resistance index, and left ventricular stroke work index.

4.2. Methods

4.2.1 Study design

We report on two successive studies, with the aim of establishing a clinical anesthetic maneuver that reliably yields a target whole blood propofol concentration of 5 µg/mL. The first study (Study 1) was an open label pilot dose finding study in 24 patients who received one of three propofol doses by continuous infusion during CPB. Propofol concentrations were mathematically described as a function of the infusion rate with an empirical line of best fit, constructed using nonlinear regression followed by Akaike’s Information Criteria comparison. The pharmacokinetics of propofol are most accurately described by a three compartment model\textsuperscript{454}. True pharmacokinetic steady state for propofol under a 3-compartment model would require in excess of 3 days for non-obese patients, and beyond 10 days for obese patients\textsuperscript{455}. Given these timescales, it is unreasonable to anticipate steady state conditions for propofol during cardiac surgery –let
alone during the ischemic or reperfusion phase. For these reasons, the mathematical function describing the relationship between the infusion rate and the propofol concentration achieved at reperfusion was not modeled on pharmacokinetic or physiological principles. We used this mathematical function solely to determine the infusion rate predicted to yield our target propofol concentration.

The infusion rate derived from Study 1 was employed in a subsequent and ongoing randomized controlled trial, entitled PRO-TECT II (www.clinicaltrials.gov NCT00734383, see Chapter 5). We planned an analysis of propofol concentrations at the midpoint of the PRO-TECT II trial (n=72) in those patients randomized to the propofol treatment arm (n=30). The purpose of this interim analysis (Study 2) was to assess the reliability with which our clinical maneuver achieves our target propofol concentration at reperfusion.

Both studies focus on propofol concentrations in whole blood sampled 15 minutes after reperfusion in vacutainer tubes containing EDTA as the anticoagulant (Becton Dickinson, NJ). This operative timeframe precludes pharmacokinetic analysis. Hemodynamic measures of cardiac index, systemic vascular resistance index, and left ventricular stroke work index were also recorded prior to CPB, upon separation from CPB, and prior to patient transfer to the intensive care unit in both part 1 and in part 2.

4.2.2 Study population

This investigation conforms to the principles outlined in the Declaration of Helsinki. Following institutional approval and informed patient consent, we enrolled hemodynamically stable patients scheduled for revascularization of 3 or more coronary
vessels where a minimum continuous aortic crossclamp time of 60 minutes was anticipated. We excluded patients who: 1) were less than 18 or greater than 80 years of age; 2) refused consent; 3) had co-existing valvular heart disease; 4) had an acute or evolving myocardial infarction; 5) had a history of hypersensitivity to propofol or any formulation component.

4.2.3 Perioperative procedures

Perioperative monitoring (arterial, central, and pulmonary catheterization), surgical, and cardioplegia techniques (warm, intermittent, antegrade delivery of blood:crystalloid (8:1 ratio)) were standardized. Tranexamic acid $0.05 \, \text{mg/kg}$ then $0.10 \, \mu g \cdot kg^{-1} \cdot min^{-1}$ was the antifibrinolytic therapy of choice. CPB was conducted at $34-37^\circ C$. Intraoperative hematocrit was maintained at 0.25 to 0.27 during CPB and facilitated by retrograde autologous prime procedure$^{528}$.

4.2.4 Anesthesia protocol

Anesthesia was standardized to induction with fentanyl 10-15 $\mu g/kg$, midazolam 2-4 mg and sodium thiopental as required for loss of consciousness. Muscle relaxation and tracheal intubation were achieved with rocuronium 0.1 mg/kg. Anesthesia was maintained with isoflurane (0.5-2%, end tidal), except during CPB when propofol was administered. Post-CPB anesthesia was as per clinical practice of the attending anesthesiologist.
4.2.5 Application of propofol during cardiopulmonary bypass

Delivery of isoflurane was discontinued approximately ten minutes prior to aortic crossclamp. Propofol was then applied as a 1.0 mg/kg bolus followed by a continuous infusion of 50 (n=8), 100 (n=9) or 150 µg•kg\(^{-1}\)•min\(^{-1}\) (n=7) in Part One, or 120 µg•kg\(^{-1}\)•min\(^{-1}\) (n=30) in Part Two, until 15 minutes after release of the aortic crossclamp (reperfusion).

4.2.6 Measurement of propofol concentration

Four milliliters of whole blood was sampled from the central venous line 15 minutes after reperfusion to accommodate the surgeon after crossclamp removal. Whole blood was sampled using vacutainer tubes containing EDTA as the anticoagulant (Becton Dickinson, NJ), then stored as ~1.25 mL aliquots and stored at -80°C for subsequent quantitative propofol analysis by capillary electrophoresis\(^{529}\) (see Chapter 3).

4.2.7 Hemodynamic data collection

Intraoperative central venous pressure and mean pulmonary catheter wedge pressure were maintained to within ±20% of baseline values by volume transfusion from the CPB reservoir. Transesophageal echocardiography was employed during the perioperative period to facilitate volume loading, and to rule out cardiac tamponade, pneumo- or hemothorax as possible causes of cardiac depression. Intraoperative cardiac function (cardiac index, systemic vascular resistance index, left ventricular stroke work index) was measured and derived at three timepoints: pre-CPB, post-CPB emergence, and just prior to admission to the intensive care unit (pre-ICU).
4.2.8 Inotropic and vasoactive drug protocol

Intraoperative hemodynamic management included use of phenylephrine (1-2 µg/kg prn) for blood pressure below 85 systolic or mean arterial pressures below 50 mmHg. Arterial blood pressure greater than 140 mmHg systolic or mean arterial pressures above 80 mmHg was treated by deepening anesthesia by using fentanyl (1-2 µg/kg) followed by the vasodilator of choice prn at the discretion of the attending anesthesiologist. If pre-CPB heart rate was above 85 beats per minute, and if the attending anesthesiologist felt that adequate anesthesia and analgesia had been achieved, patients were treated with metoprolol intravenous prn.

Systolic blood pressure less than 90 mmHg in the presence or absence of a cardiac index below 2.2 L•min⁻¹•m⁻², despite a pulmonary capillary wedge pressure range of 12 to 15 mmHg at the time of separation from CPB, was treated with dopamine or dobutamine (> 4 µg•kg⁻¹•min⁻¹), epinephrine or norepinephrine (> 0.04 µg•kg⁻¹•min⁻¹), alone or in combination with milrinone (0.25 to 0.75 µg•kg⁻¹•min⁻¹) at the discretion of the attending anesthesiologist. Inotropic support exceeding 30 minutes in duration under the conditions described in Table 6 was considered clinically significant.
**Table 6:** Suggested dose regimen for attending anesthesiologists.

<table>
<thead>
<tr>
<th>Inotrope</th>
<th>Starting Dose</th>
<th>Dose Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>epinephrine</td>
<td>0.5 to 2 µg/min</td>
<td>0-8 µg/min</td>
</tr>
<tr>
<td>milrinone</td>
<td>0.125-0.25 µg•kg⁻¹•min⁻¹</td>
<td>0-0.75 µg•kg⁻¹•min⁻¹</td>
</tr>
<tr>
<td>dobutamine</td>
<td>3.5-7.5 µg•kg⁻¹•min⁻¹</td>
<td>0-10 µg•kg⁻¹•min⁻¹</td>
</tr>
<tr>
<td>dobutamine</td>
<td>1.5 to 3.5 µg•kg⁻¹•min⁻¹</td>
<td>0-10 µg•kg⁻¹•min⁻¹</td>
</tr>
</tbody>
</table>

*SVR<600*

<table>
<thead>
<tr>
<th>Inotrope</th>
<th>Starting Dose</th>
<th>Dose Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>norepinephrine</td>
<td>2-4 µg/min</td>
<td>0-8 µg/min</td>
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</table>

*SVR>1200*

<table>
<thead>
<tr>
<th>Inotrope</th>
<th>Starting Dose</th>
<th>Dose Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>milrinone</td>
<td>0.125-0.75 µg•kg⁻¹•min⁻¹</td>
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</tr>
</tbody>
</table>

*mPAP>25*

<table>
<thead>
<tr>
<th>Inotrope</th>
<th>Starting Dose</th>
<th>Dose Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG and/or milrinone</td>
<td>0.125-0.75 µg•kg⁻¹•min⁻¹</td>
<td></td>
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</tbody>
</table>

SVR: systemic vascular resistance, mPAP: mean pulmonary arterial pressure, NTG: Nitroglycerine.

**4.2.9 Sample size and statistical analysis.**

Based on two of our previous studies\textsuperscript{409,410}, we anticipated a one-tailed difference in whole blood propofol concentrations of 2.2 µg/mL between doses in study 1, with a standard deviation of 1.4 µg/mL. The type 1 error rate was set at $\alpha=0.05$ and the power at 0.9. Accordingly, we determined a minimum sample size of seven patients per group. At the midpoint of PRO-TECT II, 72 patients had been randomized, 30 to the propofol treatment arm. This comprises the sample available for analysis for Study 2; no formal sample size calculation was performed.
All data are reported and presented as the mean and standard deviation except for predicted values and constant of proportionality, which are described using 95% confidence intervals. Hemodynamic parameters from Part 1 were analyzed using a two-way repeated-measures ANOVA. Bonferroni/Dunn post-tests for pair-wise comparisons of averages for doses across time were performed when the variance of the dose-time interaction reached a significance level of $p \leq 0.05$. Hemodynamic parameters from Part 2 are presented descriptively. All analyses were performed using GraphPad Prism 4.0c software.

4.3. Results

4.3.1 Patient and operative characteristics

Patient and operative characteristics according to experimental group are described in Table 7. Insufficient anesthesia, as evidenced clinically by elevated mean arterial pressure (exceeding 80 mmHg) and low mixed venous oxygenation (less than 65%) on CPB, was suspected by the attending anesthesiologist in three patients who had received a propofol infusion of $50 \, \mu g \cdot kg^{-1} \cdot min^{-1}$ during CPB. These patients received supplemental isoflurane. Two patients receiving a propofol infusion of 150-, and one receiving a propofol infusion of $100 \, \mu g \cdot kg^{-1} \cdot min^{-1}$, were described as clinically unstable at separation from CPB. They required two or more inotropes, alone or in combination with norepinephrine, for hemodynamic stabilization prior to intensive care unit transfer.
Table 7: Patient demographic and perioperative characteristics.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>50 µg•kg⁻¹•min⁻¹</th>
<th>100 µg•kg⁻¹•min⁻¹</th>
<th>150 µg•kg⁻¹•min⁻¹</th>
<th>120 µg•kg⁻¹•min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>59±7</td>
<td>70±4</td>
<td>62±10</td>
<td>63±8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.0±17.0</td>
<td>76.9±12.9</td>
<td>77.8±16.4</td>
<td>89.4±13.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162.5±8.5</td>
<td>165.7±7.3</td>
<td>166.4±9.8</td>
<td>173.1±7.2</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.78±0.23</td>
<td>1.88±0.19</td>
<td>1.86±0.24</td>
<td>2.03±0.17</td>
</tr>
<tr>
<td>Gender (m:f)</td>
<td>4:4</td>
<td>7:2</td>
<td>4:3</td>
<td>29:1</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>50±17</td>
<td>45±13</td>
<td>51±9</td>
<td>50±14</td>
</tr>
<tr>
<td>ACC (min)</td>
<td>65±29</td>
<td>101±39</td>
<td>73±20</td>
<td>89±28</td>
</tr>
<tr>
<td>CPB (min)</td>
<td>89±35</td>
<td>140±59</td>
<td>140±59</td>
<td>119±35</td>
</tr>
</tbody>
</table>

Data are expressed as mean± standard deviation or patient numbers. BSA: body surface area, LVEF: left ventricular ejection fraction, ACC: aortic cross-clamp interval, CPB: cardiopulmonary bypass interval.

4.3.2 Propofol concentrations in whole blood

Part One

The whole blood concentrations of propofol in patients treated with infusion rates of 50, 100, and 150 µg•kg⁻¹•min⁻¹ were 2.10 (1.20), 2.96 (1.87) and 14.28 (4.79) µg/mL, respectively (Figure 12). The empirical line of best fit for the relationship between propofol concentrations and infusion rates was determined using non-linear curve fitting. According to an Akaike’s Information Criteria comparison, an exponential growth non-linear model was preferred over the alternative power series model. Equation 18 describes
the model mathematically, in which the $y$-variable represents the achieved whole blood concentration, the $x$ represents the infusion rate, and $a$ (0.215; 95%CI=-0.088 to 0.519) and $K$ (0.0279; 95%CI=0.0181 to 0.0376) are constants of proportionality. The line had a coefficient of determination of $r^2=0.781$, and predicted that 113 $\mu$g•kg$^{-1}$•min$^{-1}$ was required to achieve a mean concentration of 5 $\mu$g/mL.

Equation 18: \[ y = a \cdot e^{Kx} \]

Figure 12: Propofol concentrations in whole blood at reperfusion during CABG with CPB in 24 patients receiving one of three infusion rates; 50, 100, and 150 $\mu$g•kg$^{-1}$•min$^{-1}$. The solid line represents the empirical line of best fit ($r^2=0.781$); the dotted line represents its 95% confidence interval. All concentrations were determined using capillary electrophoresis from 400 $\mu$L of whole blood, sampled 15 minutes post-reperfusion during CPB.
Part Two

There were 4 study protocol violations; two patients received Propofol 2.0 mg/kg bolus (propofol concentrations: 10.6, 11.0 µg/ml); two additional patients received no loading dose (propofol concentrations: 2.6, 2.6 µg/ml). Three cases had operative aortic crossclamp intervals below 60 minutes (propofol concentrations: 2.4, 4.0, 6.5 µg/ml), thus intraoperatively violating study inclusion criteria. These seven patients were excluded from subsequent analysis.

The whole blood propofol concentration from the remaining 23 patients was 5.39±1.45 µg/mL, with a range of 2.60 to 7.54 µg/mL. The 25%, 50%, and 75% quartiles were 4.36 µg/mL, 5.63 µg/mL, and 6.34 µg/mL, respectively.

Propofol levels were 4.45 µg/mL (25 µM) or higher in 18/23 patients (78%), and above 5 µg/mL 15/23 patients (65%). Propofol concentrations showed no apparent correlation with patient age, weight, body surface area, or aortic crossclamp duration in this series (Figure 13).

4.3.3 Intraoperative hemodynamic function

Figure 14 depicts intraoperative profiles for cardiac index, systemic vascular resistance index and left ventricular stroke work index in patients receiving propofol infusions of 50, 100, 150 µg•kg⁻¹•min⁻¹ along side those from patients receiving infusions of 120 µg•kg⁻¹•min⁻¹. We did not detect significant differences in the dose-time interaction for any hemodynamic parameters (Figure 14a,c,e). The hemodynamic profiles described in Figure 14 were similar for patients in Study 1 and Study 2.
Figure 13: Scatter plots of propofol concentration plotted against a) age, b) weight c) body surface area and d) crossclamp duration for 23 patients receiving 120 µg·kg⁻¹·min⁻¹ of propofol during CPB.
Figure 14: Intraoperative profiles of cardiac index (a,b), systemic vascular resistance index (c,d), and left ventricular stroke work index (e,f). Left panels (a,c,e) show profiles from patients receiving one of three propofol infusion rates (50, 100, and 150 $\mu$g·kg$^{-1}$·min$^{-1}$; n=24) during CPB. Right panels (b,d,f) show profiles from patients randomized to receive 120 $\mu$g·kg$^{-1}$·min$^{-1}$ of propofol (n=23) during cardiopulmonary bypass. The horizontal axis is given in terms of operative timepoints: prior to CPB (pre-CPB), on separation from CPB (post-CPB), and immediately prior to transfer to the intensive care unit (pre-ICU). Data for each treatment group are presented as the mean and standard deviation.
4.4. Discussion

The current study describes conditions under which laboratory-based propofol mediated cardioprotection was translated to an experimental clinical maneuver. In order to minimize alterations to the operative procedure, and to facilitate clinical investigation, the method employed a loading bolus followed by constant infusion focused to the CPB interval. The primary research question relates to whether cardioprotective concentrations could be reliably achieved in vivo without undue risk of cardiac depression. The principle findings of this study are: 1) the constant infusion rate predicted to achieve a mean propofol concentration of 5 µg/mL in whole blood was 113 µg•kg⁻¹•min⁻¹; 2) the propofol concentration achieved with the nearest practical rate of 120 µg•kg⁻¹•min⁻¹, was 5.39 (1.45 µg/mL), with quartiles of 25% = 4.36 µg/mL; 50% = 5.63 µg/mL; and 75% = 6.34 µg/mL. Our model predicted a concentration of 6.10 (± 1.76) µg/ml at this infusion rate; 3) patient age, weight, body surface area, or aortic crossclamp duration were not found to influence propofol concentration at reperfusion; 4) There was no evidence of depressed left ventricular function at emergence from CPB in patients receiving 120 µg•kg⁻¹•min⁻¹ propofol infusions during CPB.

The dosing groups in Part One of our study were partly modeled after a pharmacokinetic study, conducted in a nonsurgical setting, by Gepts et al. The mathematical model we used represents an empirical means to fit our data in order to predict the infusion rate most likely to produce a given propofol concentration under similar operative and anesthetic conditions. As a result, a 1 mg/kg propofol bolus followed by a 120 µg•kg⁻¹•min⁻¹ continuous infusion was chosen for our PRO-TECT II
protocol. Given its primary importance to tissue injury and repair, our sampling coincides with the early stage of reperfusion.

The method of drug application in our study produced a wide range of blood concentrations for a given infusion rate. This variability appears to be in line with that of several other studies where propofol concentrations were measured under similar operative conditions\textsuperscript{452, 456, 531-533}. It is clear that steady state conditions were not achieved, but it is also clear that steady state conditions for propofol cannot reasonably be anticipated within the context of cardiac surgery. We suggest that a significant reduction in the variance of propofol concentrations in the absence of steady state conditions will require monitoring of the concentration achieved during the course of surgery. By extension, drug level monitoring may be required to appropriately evaluate the role of propofol in cardioprotection, and its absence in experimental clinical studies makes interpretation of findings difficult.

We are satisfied that the experimental clinical maneuver derived in this study is capable of producing a propofol concentration associated with laboratory based cardioprotection. Indeed, the effect of increased propofol dosing to achieve the therapeutic concentration range associated with laboratory based propofol mediated cardioprotection (25-50 µM) was achieved clinically in 78% of patients in our study. The highest level we measured was approximately 7.5 µg/ml (45 µM) as seen in 17% of cases. This concentration is clinically and experimentally relevant, given these levels have been previously associated with the range expected to inhibit both lipid peroxidation\textsuperscript{487} and mitochondrial permeability transition\textsuperscript{521}. The absence of high drug levels among patients in study 2 suggests that a large dose of propofol, applied during
CPB, has no detrimental effect on early post-bypass functional recovery relative to lower infusion rates. This contrasts with reports where total intravenous anesthesia with propofol and remifentanil was used for cardiac surgery\textsuperscript{375}.

We did not observe a decrease in cardiac index, consistent with our definition of cardiac depression, upon emergence from CPB across dosing groups. This pattern was associated with a decrease in systemic vascular resistance index and the maintenance of left ventricular stroke work index (Figure 14). By extension, elevated doses of propofol during cardioplegic arrest do not appear to increase the risk of cardiac instability on emergence from CPB. The benefit of this method with respect to clinical outcomes and cardioprotection cannot be extrapolated in the current study, and remains to be determined.

Patient characteristics of age, disease state, and weight have been identified as significant covariates that influence propofol pharmacokinetics\textsuperscript{454,530}. Their effect on data spread effects is likely to be amplified in non-steady state conditions. We did not find any systematic influence of these parameters on propofol concentrations at reperfusion (Figure 13), suggesting that non-steady state conditions and variability in total infused drug volume have a larger influence.

We used capillary electrophoresis to quantitatively analyze propofol in whole blood\textsuperscript{529} (see Chapter 3). The separation is completed in less than 8 minutes, but the length of the preparative step still precludes its use for point of care target-achieved type dosing. Quantitative analysis that provides target-achieved drug infusion would likely facilitate perioperative care of high-risk patients. Technologies that enable target-
achieved dosing could then be adopted for routine use in studies designed to determine clinical outcomes.

There are limitations to the present study. Propofol concentrations were only measured in central venous blood collected at one time point, which limits any pharmacokinetic interpretations of the data. Central venous sampling was used for quantitative propofol analysis. Site-effect studies have confirmed that venous sampling is equally representative of arterial drug concentrations provided the infusion interval prior to sampling is longer than 20 minutes\textsuperscript{453, 454, 534-537}. Secondly, the mathematical model described in this study is inherently susceptible to changes in the anesthetic maneuver, and is incapable of predicting propofol concentrations in routine clinical practice, or beyond the infusion rates used in our study. In the absence of controls that omit propofol anesthesia, we are unable to attribute either the magnitude or the pattern of hemodynamic changes to the administration of propofol. The volume of propofol delivered in our study prior to sampling is entirely dependent on patient weight and crossclamp interval. Crossclamp intervals are neither consistent between surgical cases nor sufficient to establish near-steady state pharmacokinetic conditions\textsuperscript{455, 530}. For these reasons, our line of best fit has no pharmacokinetic basis, its constants are not known to represent any physiological parameters, and there is no known basis for the apparent log-linear relationship between the infusion rate and the concentration that equation 18 suggests. The current study focused on the intraoperative interval. Any patterns of hemodynamic performance are not known to extend to the postoperative period. Finally, our hemodynamic findings are not known to apply to patients with severe ventricular
dysfunction and profoundly low cardiac output, or to patients treated with drugs used to treat low cardiac output, such as milrinone.

The current study introduces an experimental clinical maneuver focused to the CPB interval, capable of yielding an elevated propofol concentration at reperfusion. In summary, the administration of a 1 mg/kg bolus dose of propofol followed by a continuous infusion of 120 µg•kg⁻¹•min⁻¹ during CPB produced relevant cardioprotective drug concentrations in whole blood at reperfusion. These concentrations were associated with an increase in cardiac index at emergence from CPB, in the absence of additional inotrope support. The achieved drug concentrations have previously been associated with enhanced red cell and tissue antioxidant capacity in vitro and in vivo, reduced dysfunction subsequent to experimental ischemia-reperfusion injury, and reduced endothelial and cardiomyoblast apoptosis. Failure to prevent cardiac injury with conventional propofol doses could be explained by inadequate concentrations and timing of administration. It remains to be determined if achieving a target concentration of 5 µg/ml will improve clinical outcomes (morbidity and mortality) in high-risk patient populations undergoing cardiac surgery.
5. Rationale and design of the PROpofol Cardioprotection for Type II Diabetics (PRO-TECT II) Study: A randomized, controlled trial of high-dose propofol versus isoflurane preconditioning in patients undergoing on-pump cardiac surgery

5.1. Introduction

Diabetic patients are up to five times more likely to develop cardiovascular disease\textsuperscript{16}. Unsurprisingly, these patients account for nearly a third of CABG surgeries\textsuperscript{21}. Following cardiac surgery, individuals with diabetes suffer higher rates of perioperative morbidity and mortality, recurrence of angina and lower long-term survival rates compared to patients without diabetes\textsuperscript{12-14, 427, 428}.

In particular, diabetic patients are at elevated risk for low cardiac output syndrome\textsuperscript{14, 15}, defined as persistent hypotension (systolic blood pressure < 90 mmHg) and/or low cardiac output (cardiac index < 2.2 L min\textsuperscript{-1} m\textsuperscript{-2}) despite hemodynamic optimization. Prolonged use of high doses of inotropes, vasopressors, and/or intra-aortic balloon counterpulsation are required. The causes of low cardiac output syndrome may include the injury that follows ischemia and reperfusion of the heart and inadequate revascularization. The diabetic heart is more sensitive to this form of injury due to defective antioxidant defenses\textsuperscript{17, 18}, increased oxidative stress, and impaired endogenous myocardial protective pathways\textsuperscript{19, 20}. If inadequately treated, low cardiac output syndrome can quadruple the overall mortality rate for CABG surgery\textsuperscript{427}.

Major efforts have focused on increasing the myocardial tolerance to ischemia (preconditioning) via physical (intervals of ischemia) or pharmacological (volatile
anesthetics) means\textsuperscript{332, 516, 539-541}. The myocardial mitochondrial ATP-regulated $K_{\text{ATP}}$ channel is essential for protection by preconditioning\textsuperscript{338, 339}. Unfortunately in diabetes, signal transduction pathways required for ischemic or anesthetic preconditioning are corrupted\textsuperscript{19, 20} and sulphonylurea oral hypoglycemic agents can block $K_{\text{ATP}}$ channel opening\textsuperscript{359}. Preconditioning is insufficient to prevent injury in the context of prolonged ischemic intervals (greater than 25 to 30 min)\textsuperscript{516}. Such circumstances require a different therapeutic approach.

Elevated oxidative stress occurs during hyperglycemia and during myocardial ischemia and reperfusion. Oxidative stress promotes the conversion of NO$^\cdot$ to ONOO$^-$ and stimulates tumor necrosis factor-α (TNFα)\textsuperscript{542}, which in turn inhibits cardioprotective endothelial NOS (eNOS)\textsuperscript{543, 544} and enhances endothelin-1 (ET-1) formation\textsuperscript{545}. These factors cause cardiac dysfunction. Effective antioxidant intervention during ischemia and reperfusion appears important for preserving myocardial function. Thus, rather than increasing the myocardial tolerance to ischemia, we have focused on alleviating oxidant-mediated post-ischemic injury by increasing antioxidant defenses (cardioprotection).

Our previous work suggests that propofol, an intravenous anesthetic with antioxidant potential, may confer cardioprotection\textsuperscript{410, 414, 482, 483}. Although conventional low doses of propofol have not been clinically effective in reducing postoperative cardiac injury or improving cardiac function, \textit{in vitro} dose-finding and animal based studies from our laboratory suggest that high doses of propofol can reach a therapeutic concentration (≥10 to 25 µmol/L) needed for cardioprotection\textsuperscript{483, 519, 546}. We have translated our approach of applying high-dose propofol in the laboratory into a safe experimental maneuver during cardiac surgery.
This paper describes the design of the PROPofol Cardioprotection for Type II Diabetics (PROP-TECT II) Study, a Phase II randomized controlled trial designed to explore the relationships of biomarkers of oxidative or nitrosative stress in diabetes, determine the effect of high dose propofol cardioprotection to counteract these effects in patients undergoing elective primary CABG with CPB, and provide feasibility and sample size data needed to conduct Phase III RCTs.

5.2. Materials and methods

5.2.1 Study design

The PROP-TECT II Study is a Phase II RCT comparing high-dose propofol cardioprotection versus isoflurane preconditioning in diabetic and nondiabetic patients at risk of an adverse perioperative cardiac event who are undergoing CABG surgery requiring extracorporeal circulation. Participants, health care providers, investigators, data collectors, and laboratory staff are blinded to whether patients receive propofol or isoflurane.

5.2.2 Study population

Adult patients undergoing cardiac surgery at the Vancouver General Hospital are eligible if they are 18-80 years of age, are undergoing primary CABG surgery requiring CPB, require revascularization of three or more coronary vessels with an anticipated aortic cross clamp time of ≥ 60 minutes, and have a preoperative systolic blood pressure > 90 mmHg in the absence of inotropic or mechanical support. Patients are ineligible if they have co-existing valvular heart disease, an acute or evolving myocardial infarction, or a history of hypersensitivity to propofol or any formulation component; or are taking
non-steroidal anti-inflammatory drugs, vitamin C, or vitamin E within five days of surgery.

5.2.3 Randomization

Subjects are randomly allocated to either the propofol group or the isoflurane group after written informed consent. The allocation process uses a computer-generated random number table, with random permuted blocks of four or six, stratified by diabetic status and left ventricular ejection fraction as diabetes mellitus and decreased left ventricular function may affect the incidence rate of low cardiac output syndrome. For diabetic status, the two strata are no diabetes mellitus, defined as no history or diagnosis of diabetes mellitus, and Type II diabetes mellitus, defined as an established history and diagnosis of adult-onset diabetes mellitus treated with oral hypoglycemic agents (regardless of insulin use). For left ventricular ejection fraction, the two strata are normal, defined as a preoperative ejection fraction of at least 45% on angiography, and low, defined as a preoperative ejection fraction of less than 45% on angiography. The randomization scheme is unavailable to individuals involved in the recruitment, data collection, or management of the subjects.

5.2.4 Study protocol

Standardized anesthetic techniques are used at the Vancouver General Hospital. Intra-arterial blood pressure monitoring, central venous and pulmonary artery catheterization, and transesophageal echocardiography are used in addition to routine monitors. Subjects will undergo intravenous (IV) anesthetic induction with fentanyl 10-15 µg/kg, midazolam 0.15-0.25 mg/kg, and sodium thiopental 1-2 mg/kg followed by muscle relaxation using
rocuronium 1-2 mg/kg to facilitate tracheal intubation. Prior to CPB, anesthesia will be maintained with isoflurane 0.5 to 1.5% (end tidal). Subjects will receive phenylephrine (1-2 µg/kg), increased anesthetic depth, fentanyl (1 to 2 µg/kg), or vasodilator therapy (e.g., nitroglycerin 0.125 to 0.25 µg•kg\(^{-1}\)•min\(^{-1}\)) to maintain their systolic and mean arterial blood pressures between 85 to 140 mmHg and 50 to 80 mmHg respectively. Subjects will receive metoprolol if their pre-bypass heart rates exceed 85 bpm. Tranexamic acid (0.05 mg/kg then 0.01 mg•kg\(^{-1}\)•hr\(^{-1}\)) is the antifibrinolytic of choice, to reduce the risk of bleeding.

Following a median sternotomy, the left and right internal mammary and radial arteries will be dissected for grafts depending on the location of the coronary artery disease. Subjects will receive intermittent, antegrade blood cardioplegia during continuous aortic cross clamping. The temperature of the cardioplegia will be left to surgical preference. CPB will be conducted at 34-37°C. Intraoperative hematocrit will be maintained between 0.25 and 0.27 during CPB.

For subjects allocated to propofol cardioprotection, isoflurane will be discontinued ten minutes before CPB. At this time, subjects will receive propofol 1 mg/kg intravenous as a bolus followed by an intravenous infusion at 120 µg•kg\(^{-1}\)•min\(^{-1}\) until 15 minutes after release of the aortic cross clamp (reperfusion). For subjects allocated to isoflurane preconditioning, subjects will receive a preconditioning dose of isoflurane 2.5% (end-tidal) for ten minutes before CPB, followed by isoflurane 0.5-1.5% (end-tidal) during and after CPB, without administration of propofol. Figure 15 outlines the study interventions.
Figure 15: Representative diagram of study interventions applied during coronary artery bypass graft surgery in the PRO-TECT II Study. *ET: end tidal, IV: intravenous, CPB: cardiopulmonary bypass.*

At the end of surgery, all subjects will transfer to the cardiac surgery intensive care unit for postoperative management. Subjects will be mechanically ventilated initially after surgery until they meet criteria for weaning and tracheal extubation. Subjects will receive inotropic support, based on standard guidelines, at the time of separation from CPB and at any time in the first 24 h after surgery when the systolic blood pressure is <90 mmHg and/or a cardiac index is <2.1 L•min⁻¹•m⁻² despite pulmonary capillary wedge pressure and/or central venous pressure being 12–15 mmHg. The use of dopamine or dobutamine >4 µg•kg⁻¹•min⁻¹, epinephrine or norepinephrine >0.04 µg•kg⁻¹•min⁻¹ or milrinone 125 µg•kg⁻¹•min⁻¹, alone or in combination, for greater than 30 min will be considered clinically significant. Subjects will receive a continuous intravenous infusion
of insulin as needed to maintain glucose levels from 8 to 12 mmol/L during and after surgery.

For postoperative analgesia, subjects will receive intravenous opioids in the first 24 to 72 h postoperatively, as required. After 24 h, once oral or nasogastric intake is tolerated, subjects will switch from intravenous opioids to oral hydromorphone, oxycodone, or codeine with acetaminophen. To reduce the risk of graft occlusion, aspirin is started one day after surgery. As the antiplatelet effect of nonsteroidal anti-inflammatory drugs could confound the results of our primary outcome (see below), this class of drugs will not be prescribed for adjunctive analgesia in this study.

5.2.5 Blinding

An anesthesiologist (staff or fellow), who will be uninvolved with the clinical care of the subject, will receive the allocation by telephone from the recruiting nurse, and will initiate the intervention to maintain blinding of the subject, attending anesthesiologist, surgeon, nursing staff, and investigators. Ten minutes before CPB, the same individual will place opaque drapes over the anesthetic vaporizers and discontinue the anesthetic concentration readout on the anesthetic machine and the CPB machine to avoid unmasking of the allocation. During CPB, the unblinded study anesthesiologist, instead of the attending anesthesiologist, will direct the unblinded perfusionist in the administration of the anesthetic from the CPB machine. The drapes will be removed after separation from CPB. Patients in the isoflurane group received a mock intralipid infusion, delivered to an empty plastic bag that remained hidden from view, to mimic the propofol infusion.
Unblinding rules apply if a serious adverse event (anaphylactoid or anaphylactic reaction) occurs during the course of the study. The Principle Investigator will report the occurrence of this serious adverse event to the institutional Ethics Committee and the Health Protection Branch of Health Canada.

5.2.6 Outcome measures

The primary outcome will be the myocardial-derived plasma free 15-F2t-isoprostane level, which will be the calculated difference between the measured coronary sinus plasma level of 15-F2t-isoprostane from coronary sinus blood sampled at 5 min after release of the aortic cross clamp. Secondary biochemical outcomes include plasma total antioxidant concentration, systemic and coronary sinus levels of troponin I, ET-1, TNFα, and 3-nitrotyrosine as evidence of ONOO⁻ formation in blood; gene and protein expression of inducible NOS (iNOS) and eNOS, protein expression of Akt and its activation, and evidence of superoxide formation in atrial tissue.

The clinical outcome is the incidence rate of low cardiac output syndrome during the first 6 h after surgery. Low cardiac output syndrome is defined as a systolic blood pressure less than 90 mmHg and/or cardiac index less than or equal to 2.1 L•min⁻¹•min⁻² despite central venous or pulmonary artery occlusion pressure and/or central venous pressure of 12 to 15 mmHg, systemic vascular resistance 800 to 1200 dyne•s/cm⁵, and a spontaneous or atrioventricular-paced heart rate greater than 80 beats per min. Low cardiac output syndrome will be adjudicated in a blinded fashion. Since the adequacy of revascularization could impact our primary and secondary outcomes, the vessel diameters, vessel quality and adequacy of revascularization will be documented. We will confirm adequate filling volume using transesophageal echocardiography to continuously
assess left ventricular function during the intraoperative period and to rule out other causes of postoperative cardiac depression (e.g., cardiac tamponade, pneumothorax, hemothorax). The incidence rate of inotropic support or intra-aortic balloon counterpulsation required to treat low cardiac output syndrome for more than 30 min duration, the intensive care unit length-of-stay, and the hospital length-of-stay will be recorded.

5.2.7 Ethical considerations

This investigation conforms to the principles outlined in the Declaration of Helsinki. This study was approved by the University of British Columbia Clinical Research Ethics Board. The PRO-TECT II study is registered at www.clinicaltrials.gov (NCT00734383).

5.2.8 Sample size

Based on our previous work in humans undergoing CABG surgery and CPB using isoflurane anesthesia, plasma free 15-F_{2t}-isoprostane levels at ten minutes reperfusion was 160.7±120.4 pg/mL (mean ± standard deviation). In our previous rat studies, plasma free 15-F_{2t}-isoprostane levels decreased 70% during reperfusion (relative difference)\(^{483}\). We anticipate a 25% decrease in humans. Based on a type I error rate of 0.05, a power of 0.80, as well as an anticipated relative difference of 25% between groups with an estimated plasma free 15-F_{2t}-isoprostane level to be 200 pg/ml at 5 minutes reperfusion in the isoflurane group, the required sample size would be 36 subjects per strata per group; therefore, 144 subjects will be required.
5.2.9 Data analysis

We will use the intention-to-treat principle for all our analyses. We will describe normally distributed continuous data and skewed continuous data using the mean with its standard deviation, and medians and ranges respectively and categorical data using counts, proportions, and percentages and their 95% confidence intervals. Comparisons of levels of biochemical factors will use two-way ANOVA with a Bonferroni correction for between-group comparisons and one-way repeated measure ANOVA and Tukey's multiple comparison test for within-group comparisons. The correlations between 3-nitrotyrosine, 15-F_{2t}-isoprostane, ET-1 and cardiac index and/or left ventricular ejection fraction will be evaluated by the Pearson test. All statistical analysis will be performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

5.2.10 Funding

This project is funded with peer-reviewed grants, including the International Anesthesia Research Society Clinical Scholar Award, the Canadian Institutes of Health Research grant MOP 82757, the Canadian Anesthesiologists' Society Dr. Earl Wynands Research Award in Cardiovascular Anesthesia, the Natural Sciences and Engineering Research Council of Canada and the Vancouver Coastal Health Research Institute.

5.2.11 Trial status

The PRO-TECT II Study is currently enrolling patients at a single centre in Canada. Table 8 summarizes pre- and intraoperative data describing our first 50 patients.
**Table 8:** Demographic details of the first fifty subjects enrolled in the Pro-TECT II study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n = 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, male / female</td>
<td>48/2</td>
</tr>
<tr>
<td>Age, yr ($\mu \pm \sigma$)</td>
<td>64 ± 9</td>
</tr>
<tr>
<td>Weight, kg ($\mu \pm \sigma$)</td>
<td>80.6 ± 24.7</td>
</tr>
<tr>
<td>Height, cm ($\mu \pm \sigma$)</td>
<td>160.8 ± 43.3</td>
</tr>
<tr>
<td>Body surface area, m$^2$ ($\mu \pm \sigma$)</td>
<td>1.86 ± 0.51</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, % ($\mu \pm \sigma$)</td>
<td>52.0 ± 12.4</td>
</tr>
<tr>
<td>Left ventricular ejection fraction &gt; 45%, n (%)</td>
<td>72 (%)</td>
</tr>
<tr>
<td>Left ventricular ejection fraction 25-45%, n (%)</td>
<td>28 (%)</td>
</tr>
<tr>
<td><strong>Cardiac risk factors</strong></td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td></td>
</tr>
<tr>
<td>History of myocardial infarction, n (%)</td>
<td>40 (%)</td>
</tr>
<tr>
<td>Left main stenosis &gt; 50%, n (%)</td>
<td>38 (%)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>66 (%)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>44 (%)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>25 (%)</td>
</tr>
<tr>
<td><strong>Preoperative medication use, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>$\beta$-blocker</td>
<td>84 (%)</td>
</tr>
<tr>
<td>Calcium-channel blocker</td>
<td>20 (%)</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitor</td>
<td>62 (%)</td>
</tr>
<tr>
<td>Digoxin</td>
<td>4 (%)</td>
</tr>
<tr>
<td>Statin</td>
<td>76 (%)</td>
</tr>
<tr>
<td>Oral hypoglycemic agent</td>
<td>20 (%)</td>
</tr>
</tbody>
</table>

$\mu$ = mean; $\sigma$ = standard deviation.
5.3. Discussion

Diabetic patients are at elevated risk for low cardiac output syndrome. This high risk scenario adversely affects up to 26% of diabetic patients recovering from cardiac surgery, and low cardiac output syndrome can quadruple the overall mortality rate subsequent to CABG surgery from 2% to 8%\textsuperscript{14,15}. Aggressive hemodynamic treatments are required, but can prove inadequate. Therefore, an urgent need exists for effective forms of preemptive cardioprotection.

The PRO-TECT II Study represents a novel therapeutic approach for the prevention of myocardial ischemia–reperfusion injury in patients undergoing cardiac surgery with CPB. It is the first randomized controlled trial examining two distinct intraoperative cardioprotective strategies: the effect of intravenous anesthesia applied in an elevated dose, compared to a standardized volatile anesthetic preconditioning protocol. Our research into the therapeutic potential of propofol, an intravenous anesthetic with antioxidant properties, could have a significant impact on outcome in this select population. Given that signaling pathways for endogenous myocardial protection are corrupt and oxidant stress is elevated in diabetes\textsuperscript{17,18,436}, we will determine the effectiveness of propofol to counter these effects, reduce injury, and preserve postoperative myocardial function.

The dosing, delivery, and timing of propofol administration in our study is distinct from earlier clinical preconditioning studies conducted under conditions of hypothermic crystalloid cardioplegic arrest and CPB\textsuperscript{375,376}. Our experimental intervention is designed to combine continuous drug delivery via the systemic circulation during perfusion, and intermittent delivery via blood cardioplegia during global myocardial ischemia, at
normothermia. We believe that unconventional large dosing of propofol during the critical interval of ischemia and early reperfusion is a requirement to achieve drug concentrations associated with protective benefit (>25 µM) during early reperfusion under conditions of CPB. Our study design is strengthened by the simulated propofol infusion in the volatile preconditioning experimental group to enhance study blinding.

The PRO-TECT II Study is not powered for clinical outcomes. We have employed stratified randomization to secure equal distribution of patients based on their diabetic status and preoperative ventricular function. It will therefore provide the prerequisite knowledge for a future randomized clinical trial powered to detect clinical outcomes in diabetic patients presenting for coronary revascularization.

In addition to providing information on cardiac injury (enzymatic myocardial infarction, incidence of low cardiac output syndrome) and the 15-F_2t-isoprostane-endothelin-1/eNOS relation to cardiac dysfunction, gene and protein expression of the Akt/eNOS cardioprotective pathway in response to propofol cardioprotection or volatile anesthetic preconditioning are of specific interest to our group. The gene and protein expression profiles that may influence cell survival versus cell death may enable the prediction of a given patient phenotype that is at increased risk of perioperative myocardial injury. Quantitative analysis of bioamines in coronary sinus blood may provide mechanistic insight to activity resulting from gene or protein activation realized in tissue. Determination of intravenous propofol levels achieved under conditions of CPB will establish the drug dosing protocols required to provide reliable delivery of cardioprotective concentrations of propofol for use in expanded clinical trials.
In conjunction with the study described above, we plan to investigate the possible role for therapeutic combination of drugs for decreasing risks of cardiac events. In clinical preconditioning studies, the effects of volatile anesthetic preconditioning have been examined with no consideration for the effects of drug combinations, including insulin, statins and β-blockers. We should be able to provide important information on a therapeutic alternative to volatile anesthetic preconditioning for diabetics. The range of data from PRO-TECT II will allow for comprehensive assessment of potential benefits and optimal therapeutic combinations for this select population.
6. **Propofol does not differ from isoflurane preconditioning in terms of 15-F_{2\alpha}-isoprostane generation during ischemia and reperfusion in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass**

6.1. **Introduction**

Coronary artery disease remains a leading cause of death in North America\(^1,2\), and accounts for a significant and increasing economic burden estimated in the billions of dollars\(^2,3\). CABG with CPB is a surgical revascularization strategy that uses healthy vessels to bypass diseased coronary arteries. CABG with CPB reduces mortality among medium- and high-risk patients\(^6,8-10,54,7\). Despite its effectiveness, this procedure is associated with an important source of intraoperative myocardial ischemia-reperfusion injury\(^512,517\). A central hallmark of ischemia-reperfusion injury is the increased generation of ROS, which begins during ischemia but culminates with a large-scale burst at reperfusion.

Several studies indicate that ischemia and reperfusion directly result in increased \(\mathrm{O}_2^-\) and \(\mathrm{NO}^-\) generation, which subsequently reacts with \(\mathrm{NO}^-\) to produce \(\mathrm{ONOO}^-\)\(^124,157,209,212\). \(\mathrm{ONOO}^-\) is readily protonated to its conjugate acid, \(\mathrm{ONOOH}\), under intracellular acidosis that develop during ischemia\(^210\). In turn, \(\mathrm{ONOOH}\) rapidly degrades into \(^\cdot\ce{OH}\) and \(\mathrm{NO}_2^-\) radicals\(^209\), both of which readily initiate free radical reactions with biomolecules. Although myocardial generation of \(\mathrm{O}_2^-\) and \(\mathrm{NO}^-\) are not restricted to ischemia and reperfusion, the generation of \(\mathrm{ONOO}^-\) and \(^\cdot\ce{OH}\) are not appreciable in its absence\(^78,124,169\).

The unsaturated fatty acids that comprise cellular and subcellular lipid membranes are particularly susceptible to self-propagating peroxidation reactions initiated by \(^\cdot\ce{OH}\). Lipid
peroxidation products can adversely affect the functional integrity of lipid membranes, and are therefore quickly cleaved and released by membrane phospholipases. One such product, 15-F₂-isoprostane, has potent dose-dependent vasoconstrictor activity in vivo and may exacerbate intraoperative ischemia-reperfusion injury. Antioxidant-based therapies have been shown to reduce markers of lipid peroxidation, but the overall benefit in terms of clinical and functional outcomes remains unclear. The ability to effectively translate these therapies appears to require that the antioxidants need to be delivered with sufficient concentrations to the myocardium at reperfusion. The phenolic structure of propofol confers this intravenous anesthetic with an antioxidant capacity. Several studies indicate that this antioxidant capacity may translate to a reduction in biomarkers of ROS-mediated lipid peroxidation in various in vivo and in vitro models of ischemia-reperfusion injury. Relatively few reports describe the antioxidant capacity of propofol from the perspective of 15-F₂-isoprostane and increased ONOO⁻ biosynthesis during ischemia and reperfusion during CPB, and even fewer measure and report the concentration of propofol that was achieved in whole blood at reperfusion.

In this study, we sought to determine if a propofol infusion during CPB with blood-cardioplegia, designed to achieve drug concentrations associated with cardioprotection in experimental models, could reduce the generation of 15-F₂-isoprostane and ONOO⁻ relative to a standardized isoflurane preconditioning and maintenance protocol (see Chapter 5). The primary outcome of this study was the measurement of 15-F₂-isoprostane, which was used as an indicator of ROS-mediated lipid peroxidation. Secondary outcomes included plasma nitrite (NO₂⁻) and serum protein-bound 3-
nitrotyrosine as markers of NO$^\circ$ and ONOO$^-$ generation, respectively. We measured intraoperative and early postoperative hemodynamic performance using cardiac index, systemic vascular resistance index, and left ventricular stroke work index in order to identify any evidence of clinically significant cardiac depression upon separation from CPB.

6.2. Methods

6.2.1 Study design

This study is a component of an ongoing Phase II randomized controlled trial, entitled PRO-TECT II (www.clinicaltrials.gov NCT00734383, see Chapter 5)$^{527}$, comparing high-dose propofol cardioprotection versus isoflurane preconditioning in diabetic and nondiabetic patients at risk of an adverse perioperative cardiac event who are undergoing CABG surgery requiring CPB at Vancouver General Hospital. Participants, health care providers, investigator, and data collectors were blinded to patient randomization until completion of all pertinent analyses.

6.2.2 Study population

This investigation conforms to the principles outlined in the Declaration of Helsinki. Following institutional approval and informed patient consent, we enrolled hemodynamically stable patients scheduled for revascularization of 3 or more coronary vessels where a minimum continuous aortic crossclamp time of 60 min was anticipated. We excluded patients who: 1) were less than 18 or greater than 80 years of age; 2) refused consent; 3) had co-existing valvular heart disease; 4) had an acute or evolving myocardial infarction; 5) had a history of hypersensitivity to propofol or any formulation component.
6.2.3 Perioperative procedures

Standardized anesthetic techniques are used at the Vancouver General Hospital. Intra-arterial blood pressure monitoring, central venous and pulmonary artery catheterization, and transesophageal echocardiography are used in addition to routine monitors. Subjects underwent intravenous anesthetic induction with fentanyl 10-15 µg/kg, midazolam 0.15-0.25 mg/kg, and sodium thiopental 1-2 mg/kg followed by muscle relaxation using rocuronium 1-2 mg/kg to facilitate tracheal intubation. All patients received isoflurane 0.5 to 1.5% (end tidal) for the maintenance of anesthesia prior to CPB. Subjects received phenylephrine (1-2 µg/kg), fentanyl (1 to 2 µg/kg), or vasodilator therapy (e.g., nitroglycerin 0.125 to 0.25 µg•kg⁻¹•min⁻¹) to maintain their systolic and mean arterial blood pressures between 85 to 140 mmHg and 50 to 80 mmHg respectively. Subjects received metoprolol if their pre-bypass heart rate exceeded 85 bpm. Tranexamic acid (0.05 mg/kg then 0.01 mg•kg⁻¹•hr⁻¹) was used as the antifibrinolytic of choice to reduce the risk of bleeding.

Following a median sternotomy, the left and right internal mammary and radial arteries were dissected for grafts depending on the location of the coronary artery disease. Subjects received intermittent, antegrade blood cardioplegia during continuous aortic cross clamping. CPB was conducted between 34-37°C. Intraoperative hematocrit was maintained between 0.25 and 0.27 during CPB.

The study protocol was initiated approximately ten minutes prior to CPB at heparinization. At this time, patients randomized to the propofol arm received an initial 1 mg/kg intravenous bolus of propofol followed by a 120 µg•kg⁻¹•min⁻¹ intravenous infusion for the duration of CPB. Patients randomized to the isoflurane arm received a
standardized preconditioning dose of isoflurane (2.5% end-tidal for ten minutes before CPB), followed by isoflurane maintenance (0.5-1.0% end-tidal) throughout CPB, without administration of propofol (Figure 15). Weaning from CPB and post-CPB anesthesia was accomplished according to the routine clinical practice of the attending anesthesiologist, who was blinded to the treatment arm during CPB.

6.2.4 Blinding

An anesthesiologist (staff or fellow), who was not involved with the clinical care of the subject, received the patient allocation by telephone and initiated the intervention to maintain blinding of the patient, attending anesthesiologist, surgical staff, nursing staff, and investigators. Ten minutes before CPB, this same individual placed an opaque drape over the anesthetic vaporizers and discontinued the anesthetic concentration readouts on the relevant instruments to avoid unmasking of the allocation. This unblinded study anesthesiologist directed the unblinded perfusionist in anesthetic administration through the CPB machine. The drapes were removed after separation from CPB. Patients in the isoflurane group received a mock intralipid infusion, delivered to an empty plastic bag that remained hidden from view, to mimic the propofol infusion.

6.2.5 Measurement of propofol concentration

Four milliliters of whole blood was sampled from the central venous line 15 minutes after reperfusion using vacutainer tubes containing EDTA as the anticoagulant (Becton Dickinson, NJ), and subsequently stored at -80°C for subsequent quantitative propofol analysis by capillary electrophoresis\textsuperscript{529} (see Chapter 3).
6.2.6  *Coronary sinus blood sampling*

Coronary sinus blood was sampled immediately prior to the initiation of CPB and 5 minutes after crossclamp removal using vacutainer tubes containing EDTA as the anticoagulant (Becton Dickinson, NJ). Samples were centrifuged (10 minutes at 10,000 rcf) and plasma was separated and stored at -80°C in 1.25ml aliquots. Samples destined for 15-F$_{2\alpha}$-isoprostane were frozen in the presence of 0.005% of butylated hydroxytoluene.

6.2.7  *15-F$_{2\alpha}$-isoprostane analysis*

15-F$_{2\alpha}$-isoprostane was quantitatively analyzed using immunoaffinity purification followed by liquid chromatography-mass spectrometry analysis. In brief, 500 µL of plasma was spiked with 15-F$_{2\alpha}$-isoprostane-D$_4$ internal standard and applied to an 8-isoprostane affinity column (Cayman Chemical, Ann Arbor, MI, USA) as previously described$^{548}$. The eluent was dried in a vacuum centrifuge, and resuspended in water containing 2% Acetonitrile and 0.01% NH$_4$OH for subsequent LC-MS analysis. Liquid chromatography was performed on a C$_{18}$ column (Phenomenex) using a 12 minute gradient, from 5% to 20% B, with the following mobile phase composition: A) 0.01% NH$_4$OH in water. B) 0.01% NH$_4$OH in Acetonitrile. Quantitative analysis was accomplished using an ion trap mass spectrometer (Bruckner Daltonics), comparing the ratio of the intensity of the signal at m/z=353.1 and m/z=357.1. Figure 16 represents a typical ion chromatogram of findings in human plasma from our lab.
Figure 16: Ion chromatogram of 15-F_{2\alpha}-isoprostane with 15-F_{2\alpha}-isoprostane-D4 internal standard in patient plasma. HPLC separation on a C18 column in tandem with ion trap mass spectrometry.

6.2.8 Nitrite analysis

The concentration of NO\textsubscript{2}\textsuperscript{-} in coronary sinus plasma was measured as a stable surrogate marker of NO\textsuperscript{*} generation. Plasma sample were deproteinized as follows: Plasma (100 µl) was supplemented with 200 µl of ethanol, vortexed, and centrifuged (13000 rcf for 5 min). The supernatant was recovered, dried using a vacuum centrifuge, and resuspended in 100 µl of ultrapure water (Cayman Chemical, MI). Samples containing NO\textsubscript{2} were refluxed in glacial acetic acid containing sodium iodide to quantitatively reduce NO\textsubscript{2} to NO\textsuperscript{*}, which was subsequently reacted with ozone and analyzed by chemiluminescence detection using an NO\textsuperscript{*} analyzer (Sievers\textsuperscript{549}).
6.2.9 Measurement of 3-nitrotyrosine generation during ischemia and reperfusion

3-Nitrotyrosine-modified serum protein was detected in coronary sinus plasma as a stable surrogate marker of ONOO⁻ generation using a competitive ELISA assay (Upstate-Milipore, MA). In brief, a 3-nitrotyrosine-bovine serum albumin standard was used as the immobilized antigen, which competes with 3-nitrotyrosine-modified serum proteins for the primary anti-nitrotyrosine IgG antibody. A second anti-rabbit antibody conjugated to horseradish peroxidase subsequently binds any bound primary antibody, whose chemiluminescence signal inversely correlates with the concentration of 3-nitrotyrosine present in plasma samples. Quantitative results are derived through the construction of a standard curve with serially diluted 3-nitrotyrosine-bovine serum albumin standards.\(^ {550} \)

6.2.10 Hemodynamic data collection

Intraoperative central venous pressure and mean pulmonary catheter wedge pressure were maintained to within ±20% of baseline values by volume transfusion from the CPB reservoir. Transesophageal echocardiography was employed during the perioperative period to facilitate volume loading, and to rule out cardiac tamponade and pneumo- or hemothorax as possible causes of cardiac depression. Intraoperative cardiac function (cardiac index, systemic vascular resistance index, left ventricular stroke work index) was measured and derived at five timepoints: pre-CPB, post-CPB emergence, on admission to the intensive care unit (o/a-ICU), and 2 and 4 hours post admission to the intensive care unit (2h ICU and 4h ICU, respectively).
6.2.11 Sample size

The power analysis for the PRO-TECT II study as a whole was based on the variation from 15-F₂₆-isoprostane results that were generated using ELISA analysis (Chapter 5). A subsequent validation study indicated that ELISA-based 15-F₂₆-isoprostane analyses are invalid substitutions for mass-spectrometry based analyses. Accordingly, liquid chromatography-mass spectrometry was adopted for the quantitative analysis of 15-F₂₆-isoprostane, including an interim analysis to approximate the variance of 15-F₂₆-isoprostane levels among patients prior to the initiation of CPB (prior to any experimental intervention). These results, derived from the revised methodology, were used verify the suitability of the power analysis using equation 19. The type 1 error rate and power were maintained at $\alpha=0.05$ ($z_\alpha=1.65$) and 0.8 ($\beta=0.2; z_\beta=0.842$), respectively, while the 15-F₂₆-isoprostane levels at baseline were $152 \pm 109$ pg/mL ($\mu \pm \sigma$) with the relative difference anticipated to attain clinical relevance set at 25%.

Equation 19:

$$n = \frac{2\sigma^2(z_\alpha + z_\beta)^2}{[\Delta \mu]^2}$$

Accordingly, the required sample size is calculated at 52 patients per group for a total of 104 patients. In anticipation of study deviations, analysis of these data would commence when 110 patients had been randomized.

6.2.12 Statistical analysis

All data are reported and presented as the mean with its standard deviation except for predicted values and the constants of proportionality, which are described using 95% confidence intervals. Post-reperfusion concentration values are multiplied by the
fractional difference in patient hematocrit in order to correct for hemodilution. Hemodynamic parameters were analyzed using two-way repeated-measures ANOVA. Bonferroni/Dunn post-tests for pair-wise comparisons of averages for doses across time were performed when the variance of the dose-time interaction reached a significance level of $p \leq 0.05$. Comparisons between anesthetic treatments were considered significant when $p \leq 0.05$. The directionality (increase or decrease) of an effect was considered significant when the 95% confidence interval of its mean failed to extend across zero. All analyses were performed using GraphPad Prism 4.0c software.

6.3. Results

6.3.1 Patient and operative characteristics

A total of 112 patients were enrolled for the current study, and allocated to either the propofol or the isoflurane arm using a computer-generated random number table within the context of the blocked randomization scheme of the PRO-TECT-II study\textsuperscript{527} (see Chapter 5). Fifteen of the 112 enrolled patients were randomized but not studied for various operative and administrative decisions made after randomization: six patients required off-pump CABG due to coexisting aortic disease, one patient required concomitant valvular surgery, one patient required a MAZE procedure, the study could not reasonably be accommodated by operating room staff in the case of three patients, and four patients were recruited immediately prior to a temporary moratorium of clinical research activities to facilitate an institutional review of an unrelated study. Of the remaining 97 patients, 46 were randomized to the propofol arm, while 51 were
randomized to the isoflurane arm (Figure 17). The demographic and perioperative characteristics for these patients are described in Table 9.

**Figure 17:** Breakdown of patient randomization and study completion. (OR denotes operating room).
Table 9: Patient demographic and perioperative characteristics.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Propofol</th>
<th>Isoflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>46</td>
<td>51</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>64±9</td>
<td>65±8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.8±14</td>
<td>83.4±17</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>171±12</td>
<td>171±9</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>2.00±0.20</td>
<td>1.95±0.23</td>
</tr>
<tr>
<td>Gender (m:f)</td>
<td>42:4</td>
<td>42:9</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>50±15</td>
<td>49±12</td>
</tr>
<tr>
<td>Diabetic (n)</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>No. of grafts (n)</td>
<td>4±1</td>
<td>4±1</td>
</tr>
<tr>
<td>ACC (min)</td>
<td>86±27</td>
<td>83±29</td>
</tr>
<tr>
<td>CPB (min)</td>
<td>112±36</td>
<td>112±45</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation or patient numbers. BSA: body surface area, LVEF: left ventricular ejection fraction, ACC: aortic cross-clamp interval, CPB: cardiopulmonary bypass interval.
6.3.2 15-F_{2\alpha}-isoprostane generation during ischemia and reperfusion

15-F\textsubscript{2}\textalpha-isoprostane increased significantly during ischemia and reperfusion among patients receiving propofol (99% CI: 0.24 to 0.41 logarithmic fold change units) and among patients receiving isoflurane (99% CI: 0.19 to 0.40 logarithmic fold change units). No significant difference was found between the groups (Figure 18a).

The generation of 15-F_{2}\textalpha-isoprostane during ischemia and reperfusion was plotted against the propofol concentration achieved in whole coronary sinus blood at reperfusion (Figure 18b). The slope of the correlation analysis line does not significantly deviate from zero ($p=0.9953$; 95% CI: -0.0257 to 0.0259; $r^2$=8.3x10^-7).

6.3.3 NO\textsubscript{2} generation during ischemia and reperfusion

NO\textsubscript{2} increased significantly during ischemia and reperfusion among patients receiving propofol (99% CI: 0.087 to 0.16 logarithmic fold change units) and among patients receiving isoflurane (99% CI: 0.117 to 0.214 logarithmic fold change units) (Figure 19). This increase is greater among patients receiving isoflurane, but not significantly so ($p=0.07$).

6.3.4 3-Nitrotyrosine generation during ischemia and reperfusion

3-Nitrotyrosine increased significantly during ischemia and reperfusion among patients receiving propofol (95% CI: 0.09 to 0.34 logarithmic fold change units), but we did not detect a significant increase among patients receiving isoflurane (95% CI: -0.19 to 0.07 logarithmic fold change units) (Figure 20). This difference in 3-nitrotyrosine generation between anesthetic treatments was found to be statistically significant ($p=0.0029$, 95% CI of the difference: 0.10 to 0.46 logarithmic fold change units).
Figure 18: Change in 15-F2t-isoprostane generation during myocardial ischemia and reperfusion a) plotted according to the anesthetic protocol that patients were randomized to, and b) plotted against the concentration of propofol achieved in whole blood at reperfusion.
Figure 19: Nitrite generation changes during myocardial ischemia and reperfusion during CABG with CPB in patients randomized to propofol or isoflurane anesthesia protocols.

Figure 20: Protein-bound 3-nitrotyrosine changes in plasma during myocardial ischemia and reperfusion during CABG with CPB in patients randomized to propofol or isoflurane anesthesia protocols.

6.3.5 Perioperative hemodynamic endpoints

Perioperative hemodynamic parameters of cardiac index, systemic vascular resistance index, and left ventricular stroke work index were not significantly different between study groups (Figure 21).
Figure 21: Perioperative profiles of cardiac index (upper panel), systemic vascular resistance index (middle panel), and left ventricular stroke work index (lower panel). Data for each treatment group are presented as the mean and standard deviation. The horizontal axis represents the operative timeframe, so the intervals between markings do not necessarily correspond to equivalent time intervals.
6.4. Discussion

The primary finding of this study is that the magnitude of 15-F₂t-isoprostane generation during ischemia and reperfusion increased to a similar extent in patients randomized to receive either propofol or isoflurane anesthesia during CPB. This suggests that neither treatment can quench ROS formation or activity at reperfusion. The secondary findings demonstrate that the level of protein-bound 3-nitrotyrosine in plasma increased during ischemia and reperfusion among patients receiving propofol, but not among those receiving isoflurane. This difference was statistically significant, but was not reflected by the pattern of NO₂⁻ generation during the same interval.

A hallmark of post-ischemic reperfusion is the transient large-scale generation of ROS originating within the cells of the reperfused tissue. This phenomenon manifests itself in the current study as the significant generation of 15-F₂t-isoprostane in coronary sinus plasma (Figure 18), and stands in agreement with several other studies that clearly indicate ROS generation during myocardial reperfusion²³⁹,²⁴⁶,⁵⁵³. The current study differs in that we compare 15-F₂t-isoprostane generation during ischemia and reperfusion within the context of CABG with CPB, where patients receive one of two potentially cardioprotective anesthesia protocols. In the first group, patients received propofol during CPB according to an experimental maneuver capable of producing drug concentrations in whole blood that reflect conditions associated with propofol mediated cardioprotection in the laboratory setting⁴¹¹ (see Chapter 4). In the second group, patients received isoflurane according to a standardized volatile anesthetic mediated preconditioning protocol.

Although •OH cannot be enzymatically degraded, and despite its much higher reactivity and cytotoxicity than either O₂•⁻ or ONOO⁻¹⁹³, it can be quenched by propofol⁴⁰⁷. If
propofol were capable of quenching ROS-mediated lipid peroxidation in the myocardium at reperfusion during CABG, we would expect the achieved propofol concentration in central venous blood at reperfusion to inversely correlate with the magnitude of 15-F_2t-isoprostane generation during ischemia and reperfusion. We did not detect any such correlation (Figure 18). Previous reports have demonstrated that isoflurane preconditioning is similarly incapable of reduce the magnitude of \( ^\cdot{\text{OH} \text{ generation at reperfusion} \text{}} \)\(^{434, 435}\). Accordingly, our finding that 15-F_2t-isoprostane was generated to a similar extent in both experimental groups suggests that neither protocol can significantly reduce or quench \( ^\cdot{\text{OH} \text{ -mediated lipid peroxidation reactions at reperfusion.} \text{}} \)

This interpretation contrasts with findings from several groups, including our own\(^{414}\), that demonstrate clear reductions in MDA generation during ischemia and reperfusion in the presence of propofol\(^{416, 417, 554, 555}\). Furthermore, our finding that plasma-bound 3-nitrotyrosine only increased among patients receiving propofol (Figure 18) was surprising considering the similar extent to which \( \text{NO}_2^- \text{ and 15-F_2t-isoprostane increased in both anesthetic groups (Figure 18, Figure 19).} \text{}} \)

\( \text{O}_2^- \text{ is rapidly converted to O}_2 \text{ and } \text{H}_2\text{O}_2 \text{ in a reaction catalyzed by the widely expressed enzyme superoxide dismutase. H}_2\text{O}_2 \text{ is then further converted to H}_2\text{O and O}_2 \text{ by catalase and by the glutathione antioxidant systems. The increased myocardial O}_2^- \text{ generation at reperfusion coincides with an increase in NO}^\cdot, \text{ thus favoring a reaction that yields ONOO}^- \text{ over dismutation}^{157}. \text{ This phenomenon has been documented during ischemia and reperfusion within the context of CPB}^{249}. \text{}} \)

\( \text{NO}^\cdot \text{ is generated by at least two distinct pathways during myocardial ischemia and reperfusion; NOS catalyzed and acid mediated NO}_2^- \text{ reduction (equation 12, equation 13)
only the former would contribute to the overall myocardial \( \text{NO}_2^- \) pool at reperfusion. A significant fraction of NO\(^\cdot\) can be assumed to react with \( \text{O}_2\cdot^- \) at reperfusion, which would subtract from the overall myocardial \( \text{NO}_2^- \) pool at reperfusion. Thus, it is reasonable to assume that the current \( \text{NO}_2^- \) results somewhat underestimate the true increase in NO\(^\cdot\) generation during ischemia and reperfusion.

NO\(^\cdot\) spontaneously reacts with oxygen and water to form nitrite (equation 9), and is further oxidized in the presence of oxyhemoproteins to nitrate (equation 10a/b\(^{155}\)). Although dietary nitrates significantly contribute to their concentration in plasma, the concentrations of nitrate and nitrite after several hours of fasting, is a good indicator of overall NO\(^\cdot\) activity\(^{156}\). In this study, plasma concentrations of nitrite were analyzed and used as an indicator of NOS-catalyzed NO\(^\cdot\) generation. For the reasons stated above, nitrites likely underestimate the true increase in NO\(^\cdot\) generation during ischemia and reperfusion. An additional potential limitation to this approach stems from the finding that oxygenated hemoglobin can promote the conversion of NO\(^\cdot\) directly to nitrate with a rate constant that effectively renders the reaction diffusion limited for free oxygenated hemoglobin (equation 20\(^{556}\)). Although this reaction is somewhat inhibited by the scarcity of free oxygenated hemoglobin, NO\(^\cdot\) is can reach its stable nitrate end product by bypassing the nitrite intermediate through this pathway\(^{556}\). To this end, approximately 95% of nitrites are oxidized in whole blood to nitrate after 1 hour\(^{557}\).

Equation 20  
\[ \text{NO}^\cdot + \text{HbO}_2^\to \text{MetHb} + \text{NO}_3^\cdot \]

In the present study, venous blood was sampled from the coronary sinus both before cardiopulmonary bypass, and 5 minutes after crossclamp removal. Blood samples were processed within ten minutes of sampling, and the plasma was immediately frozen at -
80°C. Thus, nitrite oxidation is minimized due to the inherently lower oxygenated hemoglobin content of venous blood, and due to the relatively short timeframe between sampling, red blood cell removal, and sample storage.

As an alternative, deoxygenated hemoglobin can act as reductase for nitrite to yield NO• under low oxygen tension or in the case of acidosis. Furthermore, nitrate concentrations far exceed those of nitrite, so nitrate analysis or total nitrite and nitrate analysis can therefore obscure any changes in NO• production that could be reflected by nitrite. Accordingly, Lauer et al found that nitrite analyzed from venous blood reflected acute changes in regional eNOS activity, while neither nitrate nor the combined analysis of nitrate and nitrite were capable of revealing these changes. For these reasons, we used plasma nitrite analyzed from coronary sinus samples as a marker of acute NO• production.

Since the rate of ONOO• formation depends on the product of O2•− and NO• concentrations, we anticipated that the magnitude of NO2•− generation would be echoed by the generation of 3-nitrotyrosine. Our data did not demonstrate any such correlation or pattern (Figure 19, Figure 20). One possible explanation for these results is that plasma protein-bound 3-nitrotyrosine may not uniformly represent cellular ONOO• generation.

ONOO• is relatively stable at alkaline pH, but ONOOH is a strong oxidizing agent that itself can rapidly decompose to produce NO2•− and •OH radicals. ONOO• has a pKa of 6.8 at 37°C, but has a 1.9-s half-life at pH 7.4 that permits diffusion over several cell diameters. Intracellular pH decreases during myocardial ischemia within the context of CABG with CPB. The extent of this acidosis is proportional to crossclamp time and reaches a lower plateau of pH 6. The extent of intracellular acidosis during
ischemia dictates the relative directionality of equation 13, which in turn determines the half-life and diffusion of ONOO\(^\cdot\). Thus, intracellular acidosis is proportional to intracellular ONOOH activity and decomposition, which mediate nitration of tyrosine residues on intracellular proteins. These intracellular protein-bound 3-nitrotyrosine residues are only be detectable in plasma after their release from the confines of the cell. Alternatively, reduced intracellular acidosis would stabilize ONOO\(^\cdot\), facilitating its diffusion out of the cell and increasing the likelihood that it decomposes in plasma.

Interestingly, intracellular myocardial acidosis during CABG with CPB is associated with an increased need for inotropic support, impaired cardiac function, and poorer long-term post-operative outcomes\(^{563, 564}\). Furthermore, patients with diabetes are at a greater risk for intraoperative myocardial acidosis\(^{565}\), and pH recovery after reperfusion is delayed by hyperlipidemia\(^{182}\).

Propofol preserves sodium-hydrogen exchanger activity in neurons\(^{566}\) and cardiomyocytes\(^{471}\) and appears to facilitate metabolic recovery during ischemia-reperfusion injury\(^{481, 567}\). The overall effect may account for previous findings that propofol can reduce the extent of intracellular acidosis during ischemia and can increase the rate at which intracellular pH recovers\(^{471, 566, 568}\). Within the context of the current study, our observation of increased plasma protein-bound 3-nitrotyrosine generation among patients receiving propofol despite similar NO\(_2\)\(^-\) and 15-F\(_2\)\(_\alpha\)-isoprostane generation to patients receiving isoflurane may not indicate any difference in ONOO\(^\cdot\) generation, but rather a proportional difference in intracellular ONOO\(^\cdot\) degradation. In support of this notion, intracellular 3-nitrotyrosine formation is an important step in the development of post-ischemic mitochondrial dysfunction\(^{288, 289}\) and myocyte apoptosis\(^{569}\). Furthermore,
propofol can protect cultured endothelial cells from apoptosis induced by 3-morpholino-sydnominine derived-ONOO⁻ generation. Concomitant intracellular staining for 3-nitrotyrosine would be a useful adjunct experiment to further clarify whether propofol and isoflurane induce differences in intracellular 3-nitrotyrosine generation.

Decreased intracellular acidosis may also account for conflicting previous accounts of decreased MDA among patients receiving propofol. The first steps of free-radical mediated lipid peroxidation are similar for both 15-F₂-isoprostane and MDA generation. However, MDA cleavage from lipid peroxyl intermediates is an acid catalyzed reaction whose efficiency can reasonably be expected to inversely correlate with pH. It is possible that propofol does not reduce MDA levels by reducing lipid peroxidation, but rather by decreasing the acid catalyzed cleavage of MDA from its lipid peroxide precursor, which in turn becomes reduced to more stable lipid peroxidation end products. In contrast to 3-nitrotyrosine bound to intracellular proteins, phospholipid peroxidation products are rapidly cleaved by phospholipases and released into the plasma to facilitate their detection, thus accounting for the similar 15-F₂-isoprostane generation in plasma from patients in the current study. To this end, Corcoran et al reported significantly decreased MDA generation in post-reperfusion coronary sinus plasma among patients receiving propofol during CABG, but were unable to find a similar reduction in post-operative urinary 15-F₂-isoprostane generation, and attributed the reduction in MDA generation to propofol’s ability to reduce post-ischemic inflammation. A concomitant analysis of MDA would shed further light on the discord between previous MDA results and the current 15-F₂-isoprostane findings.
In order to identify any evidence of clinically significant cardiac depression during the intraoperative and early postoperative period, we recorded hemodynamic performance using cardiac index, systemic vascular resistance index, and left ventricular stroke work index. We did not detect any differences between anesthetic groups for any of these parameters (Figure 21). Intraoperative pharmacologic management of the patients by the blinded attending anesthesiologist is designed to achieve a target hemodynamic performance, and this targeted approach likely accounts for the hemodynamic similarity between treatment groups.

Differences in inotrope use required to achieve target hemodynamic endpoints could reflect differences in intraoperative injury. Inotrope use and patient outcomes in the early post-operative window (specifically low cardiac output syndrome) are recorded within the context of the PRO-TECT-II study in its entirety. At the time of this manuscript preparation, the investigators, participants, and hospital staff remain blinded with respect to these parameters. Consequently, this study is incapable of providing insight into whether the biochemical phenotype reported on in this manuscript alters the likelihood of improved outcomes.
7. **Summary and concluding remarks**

Coronary artery disease results from the buildup of atherosclerotic plaques in the lumen of coronary arteries. Such plaques, which occlude the coronary arteries and impair myocardial perfusion, lead to angina, heart failure, arrhythmia, and infarction. Several risk factors are associated with coronary artery disease, including hypertension, diabetes mellitus, dyslipidemia, excess weight and obesity, inactivity, smoking, genetics, and stress. The majority of these risk factors are increasingly common worldwide, and coronary artery disease unsurprisingly remains the leading cause of North American death, representing its largest health-related economic burden.

Less severe coronary artery disease can be treated pharmacologically or surgically without significant differences in outcomes, but coronary artery bypass grafting clearly reduces mortality among medium- and high-risk patients, including diabetics, over non-surgical management and percutaneous interventions. Although the majority of patients undergoing surgical revascularization emerge without severe postoperative complications, a significant proportion of patients develop postoperative low cardiac output syndrome which can quadruple the overall mortality rate for CABG from 2% to 8%. Low cardiac output syndrome disproportionately affects diabetic patients who are up to five times more likely to develop coronary artery disease and who account for nearly one third of CABG procedures.

Myocardial ischemia-reperfusion injury is a major source of cardiomyocyte damage, which manifests itself in a host of clinical pathologies that include cardiac arrest, acute myocardial infarction, and postoperative low cardiac output syndrome. Accordingly, any improvement in clinical outcomes associated with these pathologies must be predicated...
on reducing the magnitude of ischemia-reperfusion injury, which by extension requires an understanding of the central role that oxygen radicals play in its development.

The research presented in this thesis described the development of a capillary electrophoresis-based technique capable of quantitative analysis of propofol in whole blood. This technique was then applied within the context of a pilot dose finding study that helped dictate the clinical maneuver most likely to deliver relevant propofol concentrations to the heart at reperfusion. An appropriate study was designed to test the following central hypothesis:

Propofol reduces the incidence of low cardiac output syndrome subsequent to CABG with CPB by decreasing the magnitude of 15-F₂t-isoprostane generation during myocardial ischemia and reperfusion.

The preliminary findings contained within this thesis indicate that propofol does not significantly reduce 15-F₂t-isoprostane generation during myocardial ischemia-reperfusion compared to a standardized isoflurane preconditioning protocol. This result suggests that neither propofol nor isoflurane can significantly quench \(^{•}\)OH formation or activity at reperfusion. Indeed, our finding that the magnitude of 15-F₂t-isoprostane generation was not inversely proportional to propofol concentration suggests that the antioxidant capacity of this drug is incapable of reducing arachidonic acid peroxidation in the myocardium during CABG with CPB.

The results of this thesis further demonstrated that 3-nitrotyrosine levels increased in coronary sinus plasma among patients randomized to the propofol treatment arm, but not among those patients randomized to the isoflurane arm. Since 15-F₂t-isoprostane is indicative of \(^{•}\)OH generation –and \(O_2^{•−}\) by extension, and since 3-nitrotyrosine is
indicative of ONOOH generation, one would expect the pattern of nitrite generation to reflect that of 3-nitrotyrosine in accordance with equation 11 of this thesis. Paradoxically, nitrite generation increased to a similar extent in both groups –with a non-significant tendency towards increased generation among patients randomized to the isoflurane treatment arm.

Previous studies have demonstrated that propofol can reduce the extent of intracellular acidosis during ischemia and can increase the rate at which intracellular pH recovers. We propose that a similar effect underscores the results in our study. Deprotonated ONOO$^-$ has a half-life of approximately 1.9 second at physiological pH, which permits diffusion down its concentration gradient over several cell diameters. If previous reports indicative of reduced intracellular acidosis extend to patients randomized to receive propofol during CABG with CPB, it is conceivable that the current 3-nitrotyrosine results do not represent an increase in ONOO$^-$ generation by the myocardium, but rather an increase in its release from the myocardium. Similarly, intracellular acidosis is expected to be more severe among patients randomized to the isoflurane arm, in whom ONOO$^-$ is fractionally more protonated, and in whom tyrosine residues of intracellular myocardial proteins would undergo reciprocally greater nitration reactions than in patients randomized to receive propofol.

The proposed interpretation lends itself mechanistically to reports that correlate myocardial acidosis with impaired outcomes in the postoperative period. Specifically, patients with diabetes are at a greater risk for intraoperative myocardial acidosis, and pH recovery at reperfusion is delayed by hyperlipidemia. These patients are also at a greater risk for developing postoperative low cardiac output syndrome.
Future studies should performed using atrial tissue biopsies to determine the extent of intracellular 3-nitrotyrosine generation. These immunohistochemical or western blot based results should be analyzed in conjunction with 3-nitrotyrosine results derived from coronary sinus plasma samples. This combined analysis would provide more definitive mechanistic insights to ROS-mediated myocardial ischemia-reperfusion injury.

The current findings conflict with previous results that demonstrate propofol-mediated MDA reductions, and propofol-mediated reductions in cardiolipin depletion. Mechanisms other than reduced \(^{•}\)OH-mediated lipid peroxidation may be responsible for these previous findings. First, cardiolipin depletion appears to be initiated during ischemia as a result of mitochondrial ROS generation, and propofol can potentially preserve cardiolipin content by signaling mechanisms that are orthogonal to its antioxidant effect. Second, MDA reductions in whole animal models or clinical studies may reflect reduced post-ischemic inflammation rather than a decrease in \(^{•}\)OH-initiated peroxidation reactions endogenous to the myocardium. Alternatively, several reports indicate that propofol may reduce intracellular acidosis during ischemia and promote pH recovery at reperfusion. It is conceivable that these altered conditions could differentially affect MDA and 15-F_{2\alpha}-isoprostane generation. Future analysis of MDA in coronary sinus blood from this patient cohort may further clarify these conflicting results.

The investigators, participants, and hospital staff were blinded with respect to clinical outcomes at the time of this manuscript preparation, so the central hypothesis of the clinical study that encompasses this thesis cannot be completely addressed herein. Specifically, the incidence of low cardiac output syndrome for each treatment arm remain to be analyzed. Nevertheless, the data herein do not support the hypothesis from the
perspective of 15-F$_2$-isoprostane. In more general terms, it remains unclear whether propofol can significantly improve outcomes over volatile anesthetics among patients undergoing cardiac surgery$^{382-385}$, and it seems increasingly likely that propofol and volatile anesthetics confer mechanistically distinct cardioprotective effects. The data contained in this thesis suggests that in the case of propofol, applied during CPB to a target concentration of 5 µg/ml in whole blood, this mechanism extends beyond an increased antioxidant capacity.

The research presented in this thesis describes the foundational work performed to design a clinical study. Accordingly, Chapter 3 and 4 represent the individual components that made up the groundwork for the PRO-TECT II study, which is presented in Chapter 5, while Chapter 6 provides preliminary results that address specific research questions within the context of a clinical hypothesis. From a global perspective, this thesis can serve as a general illustration of the way in which laboratory-based conditions can be translated to a clinical investigation, and may accordingly be of use to other research programs with a similar goal. The more specific strength of the data generated within the PRO-TECT II trial resides in its head-to-head comparison of two potentially cardioprotective anesthetic maneuvers, initiated immediately prior to myocardial ischemia and extending past reperfusion. In one arm, patients were randomized to receive a standardized isoflurane preconditioning protocol. In the other arm, patients were randomized to receive propofol according to a maneuver that was specifically designed to achieve whole blood concentrations that have been associated with a protective effect in the laboratory.
The PRO-TECT II, described in Chapter 5, study is a single-center trial. Intraoperative and in-hospital patient mortality and morbidity are recorded within the context of this clinical study. Unfortunately, long-term patient outcomes beyond discharge from the hospital are not recorded. Given the relatively size of the PRO-TECT II study, the limited post-operative timeframe in which patients are followed, and the low incidence of in-hospital adverse events, it is not likely that any additional mortality benefit associated with one treatment arm over another will manifest itself in a statistically significant difference. The heterogeneity of cardiac surgeons and patients who participated in the study is likely to further contribute to the variance in patient and biomarker outcomes, and thereby provides an additional hurdle. The results of this study nevertheless provide insight into the role that anesthetics play over the course of operative myocardial ischemia and reperfusion. Additionally, the results presented in this thesis and in the PRO-TECT II study may find further value within the context of a future meta-analysis or review.

Chapter 3 described the development of an electrophoresis-based technique for the quantitative analysis of propofol. This method was capable of provided a resolved and quantifiable propofol peak in less than 8 minutes. The speed of this separation is sufficient to provide feedback to clinicians, allowing them to adjust dosing in order to achieve a target concentration within the operative timeframe. Unfortunately, the sample preparation, which was not automated and which requires more than an hour and a half, precludes such intraoperative feedback. In the current study, propofol concentrations were analyzed retrospectively, but sample preparation remains a particularly significant bottleneck to intraoperative quantitative analytical feedback within and beyond the
studies presented in this thesis. The low sample volume requirement of CE-based separations may herald improved sample preparation efficiency and, with technological advances, may facilitate the development of automated sample preparation. Of equal importance, the CE-based method described in this thesis is mechanistic similar to microfluidic-based electrophoretic separations. Microfluidic devices have the potential to further decrease separation speed and to provide clinicians with an analytical device that is small and fast enough for routine intraoperative use.

Chapter 4 described a pilot dose finding study whose results helped inform the development of a clinical maneuver capable of reliably achieving propofol concentrations in whole blood associated with whole blood cardioprotection. The results of Chapter 4 clearly demonstrate the large variance of propofol concentrations that are achieved in whole blood for any given infusion rate in patients presenting for cardiac surgery. This variance, which likely stems from a combination of patient heterogeneity and the non-steady state pharmacokinetics that arise with propofol infusion times encountered during CABG with CPB, underscores the importance of devices capable of providing quantitative feedback to clinicians.

The pharmacology of cardioprotection is an important field of research both because it sheds light on the pathogenesis of ischemia-reperfusion injury and because it may reveal more targeted strategies to improve patient outcomes. The finding in Chapter 6 that patients randomized to the propofol and isoflurane arms of the study were equivalent in terms of 15-F_{2\alpha}-isoprostane generation during ischemia and reperfusion implies that neither can reduce ROS generation over this operative interval. By extension, if volatile anesthetic preconditioning translates from laboratory-based protection to the clinic, then
the lack of clear differences in patient outcomes from meta-analyses comparing volatile preconditioning with propofol anesthesia implies that both anesthetic regimens have their own distinct cardioprotective mechanism.

The mechanistic nature behind cardioprotection, whether induced by volatile anesthetics, propofol, or ischemic preconditioning, remains speculative. In the case of volatile anesthetics and ischemic preconditioning, mitochondrial $K_{\text{ATP}}$ channel activation appears to be an important intermediate step, but this does not appear to be so for propofol. The results presented in this thesis further suggest that this mechanism extends beyond a propofol-mediated increase in tissue antioxidant capacity.

Volatile anesthetic and ischemic preconditioning reduce infarct size in laboratory-based models of ischemia-reperfusion injury. This reduced infarct size is a reflection of a decrease in cardiomyocyte death. The cell-sparing effect of preconditioning is undoubtedly desirable, but cell death and infarct size may be less relevant in cardiac surgery than in the laboratory. Conversely, reversible contractile dysfunction, or myocardial stunning, which manifests itself in low cardiac output syndrome during the early post-operative period, may ultimately be a more relevant consequence of ischemia and reperfusion within the context of CABG with CPB. Accordingly, the most relevant type of cardioprotective strategies may turn out to target the preservation of contractile protein integrity and function, as well as the maintenance of high-energy phosphates in cardiomyocytes.

The activation of intracellular proteolytic MMP enzymes subsequent to ischemia and reperfusion results in the targeted degradation of contractile proteins within cardiomyocytes. Ischemic preconditioning has been associated with reduced MMP
activity, but this association has not yet been clearly established for volatile anesthetics. It is tempting to speculate that the infarct-reducing effect of preconditioning may be robust but less relevant to cardiac surgery, while preservation of contractile function, which may be more important in terms of patient outcomes, may not be induced by these agents. Further research is required to clarify the association between volatile anesthetics and MMP activity.

Modulation of the phosphotidylinositol-3-kinase/Akt pathway has recently been associated with inhibited MMP-2 activity during ischemic preconditioning. This result may reflect the finding that MMP-2 activity can be inhibited by phosphorylation. Propofol has recently been shown to increase Akt activity in cultured cells, and this may represent a mechanistic point of convergence between the potential cardioprotective effect of propofol and its ability to modulate cell signaling. Future research is required to determine whether Akt activity definitively results in intracellular MMP phosphorylation and inactivation, and whether propofol can significantly induce this effect.

A second alternative stems from propofol’s ability to pharmacologically inhibit mitochondrial permeability transition. In doing so, propofol can maintain mitochondrial integrity and function subsequent to ischemia and reperfusion, and may thus preserve myocardial ATP synthesis and contractility. Studies that determine post-ischemic cardiac work normalized to tricarboxylic acid cycle activity or O2 consumption, in the presence or absence of propofol, are warranted. Such studies should additionally investigate the influence of propofol on post-ischemic levels of high-energy phosphates in the myocardium. Combined, these studies could help tease out whether propofol can preserve
contractility by preserving mitochondrial integrity and function, or whether it does so by preserving the integrity of the myocardial contractile machinery.

Lastly, as stated in the discussion of Chapter 6, it is conceivable that propofol’s protective effect is derived, in part, from its ability to reduce the extent of myocardial acidosis during ischemia, and to accelerate pH recovery upon reperfusion. In this way, propofol may facilitate ONOO⁻ diffusion down its concentration gradient and out of the cell, while pharmacologically maintaining mitochondrial permeability transition inhibition, despite pH recovery. Future studies into the effect of propofol on intracellular pH and the effect of sustained acidosis on contractile function during ischemia and reperfusion would further clarify the role of propofol in the context of myocardial ischemia-reperfusion injury.

Mechanistically distinct cardioprotective strategies may not translate to improved outcomes for that majority of patients who emerge from cardiac surgery without any significant postoperative complications; but they have the potential to improve outcomes for those patients in whom intraoperative myocardial ischemia-reperfusion injury manifests itself in postoperative cardiac dysfunction. The challenge going forward will involve identifying those patients that are likely to be resistant to one protective strategy as well as those that are likely to benefit from one over the other. This undoubtedly requires a better mechanistic understanding not only of volatile anesthetic preconditioning and propofol-based cardioprotection, but also of myocardial ischemia-reperfusion injury.
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