THE PROTECTIVE EFFECT OF ASCORBATE AND CATECHIN AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY IN AN ISOLATED RAT HEART MODEL

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ABSTRACT

Myocardial ischemia-reperfusion (I/R) injury is an important health concern in myocardial infarction and situations such as angioplasty and cardiac surgeries. Therefore, patients and physicians need therapeutic interventions that are applicable at the time of surgery. Flavonoids and ascorbate (vitamin C) are known for their antioxidant activity and may be involved in the currently known health benefits of plant based foods and drinks. The objectives of this study were to 1) determine the extent to which ascorbate or catechin alone at levels which could be in blood after dietary supplementation, can protect myocardial tissue in the reperfusion phase of I/R injury, and 2) evaluate the possible cooperative or synergistic protective effect of ascorbate and catechin when given together. Isolated rat hearts (n=48) were perfused in the retrograde mode with modified Krebs-Henseleit buffer, and following the induction of 30 min global ischemia, ascorbate (150 µM) and/or catechin (5 µM) were added directly into the perfusate during 90 min reperfusion. To determine the histopathological features, hematoxylin and eosin (H&E) stain was used in one heart per condition; while to assess the biochemical analysis, the heart tissues were assessed for apoptosis (caspase-3 activity), oxidative stress (thiobarbituric acid reactive substances (TBARS) and total malondialdehyde (MDA) levels), and redox status (reduced and oxidized glutathione tissue levels). A comparison of IR hearts with two controls, sham (perfused for a 15 min stabilization period) and continuous perfusion (perfused for 135 min), showed in most but not all measurements that this was a suitable model of IR injury. The treatment experiments showed that 150 µM ascorbate protected the heart against lipid peroxidation and cell apoptosis by 100%, while 5 µM catechin protected by 67% and 90% respectively. No cooperative protective effect could be observed when ascorbate and catechin were used together. None of the treatments significantly affected either reduced or oxidized glutathione levels. In

conclusion, this study showed strong protection by ascorbate, which could be used in clinically relevant situations, and is the first to report the protection by catechin at this dose under conditions of myocardial ischemia-reperfusion injury.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Δψm	Mitochondrial membrane potential
·OH	Hydroxyl radical
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
cAMP	Cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
IL	Interleukin
MDA	Malondialdehyde
NAD	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
$O^{2}.^-$	Superoxide radical
$ONOO^-$	Peroxynitrite

PMSF	Phenylmethylsulphonyl fluoride
PTP	Permeability transition pore
ROS	Reactive oxygen species
IRI	Ischemia reperfusion injury
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TBARS	Thiobarbituric acid reactive substances
t-buOOH	Tert-butylhydroperoxide
TNF-α	Tumor necrosis factor-α
TXA_2	Thromboxane A_2

CHAPTER 1: INTRODUCTION

Cardiovascular diseases including coronary (ischemic) heart disease, stroke and hypertension are the leading cause of death worldwide. According to the World Health Report of the World Health Organization (WHO) 2008, cardiovascular diseases represent 30% of all global deaths (World Health Organization, 2010).

Myocardial ischemia reperfusion injury occurs in situations such as angioplasty and cardiac surgeries which in severe cases can increase the risk of heart morbidities. Ischemia is the restriction of blood flow to the heart which causes an imbalance between oxygen demand and supply. This imbalance eventually leads to damage of the cardiac tissues. Intuitively, early restoration of blood flow to the ischemic muscle would be the most effective strategy to improve the clinical outcome. Unfortunately, reperfusion which is restoration of blood supply to the ischemic myocardium can also induce injury that might be more dangerous than ischemia itself.

The two main causative factors of ischemia-reperfusion injury are hypothesized to be the accumulation of calcium in the mitochondria and the increased production of reactive oxygen species (ROS), which plays a major role in ischemia-reperfusion injury (Murphy et al., 2008).

Most of the current interventions that are being suggested to reduce myocardial ischemia-reperfusion injury are generally effective if given before ischemia (Bolli et al., 2004). However, little is known about the effectiveness of treatments if applied clinically at the time of reperfusion to reduce the outcome of ischemia-reperfusion injury.

Flavonoids and ascorbate (vitamin C) which exist extensively in fruit and vegetables are known for their antioxidant activity and they may be involved in the currently known health benefits of plant foods (Arts et al., 2005).

In several studies, flavonoids have shown promise in protecting against ischemia-reperfusion injury through different mechanisms, which include antioxidation (Schewe et al., 2008), anti-inflammatory action (Youdim et al., 2002) and vasodilation (Bell et al., 2006). Although all the previous studies have shown protection by flavonoids against ischemia-reperfusion injury either in vitro or supplemented in the diet, many have used high levels and no studies have looked at the possible synergism of ascorbate and flavonoids against ischemia-reperfusion injury.

The present study had two objectives. The first objective was to determine the extent to which ascorbate or catechin (a flavonoid in green tea and cocoa) alone, at dietary achievable concentrations, can protect myocardial tissue in the situation of ischemia-reperfusion injury. The second objective was to evaluate the effect of ascorbate and catechin when given together and the hypothesis of a possible synergistic protective effect against ischemia-reperfusion injury.

The study was performed in isolated rat hearts, where global ischemia was induced for 30 min, followed by 90 minutes reperfusion. Ascorbate and catechin were added directly to the perfusate during the beginning of reperfusion. The heart tissues were assessed for apoptosis (caspase-3 activity), oxidative stress (thiobarbituric acid reactive substances (TBARS) and total malondialdehyde (MDA)), redox status (reduced and oxidized glutathione tissue levels) and histopathological features (hematoxylin and eosin (H&E) stain).

CHAPTER 2: REVIEW OF THE LITERATURE

2.1. Ischemia-Reperfusion Injury (IRI)

Ischemia is a partial or complete restriction of blood supply to an organ which results in tissue damage due to lack of oxygen and nutrients, while reperfusion is restoration of blood supply after a period of ischemia. Ischemia-reperfusion injury is one of the most serious complications that occur during cardiac surgeries like angioplasty, coronary bypass surgery and heart transplantation (Verma et al., 2002). The two main causative factors of IRI are hypothesized to be the accumulation of calcium in the mitochondria and the increased production of reactive oxygen species (ROS), which play a major role in ischemia-reperfusion injury (Murphy et al., 2008). On reperfusion, production of reactive oxygen species may cause effects such as arrhythmias, contractility dysfunction and cardiac cell death. One of the major key elements during this process is conversion of xanthine dehydrogenase to xanthine oxidase (Halestrap et al., 2006). This process, followed by mitochondrial permeability changes, results in more generation of reactive oxygen species and cell death (Halestrap et al., 2006) as well as release of pro-inflammatory cytokines (Taqueti et al., 2006).

2.1.1. Calcium overload in IRI

During each systole, Ca^{2+} enters through the L-type Ca^{2+} channel, which results in Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum (SR). The Ca^{2+} that enters through the L-type calcium channels will be removed mainly by Na^{+}-Ca^{2+} exchange (NCX), with a little support from the sarcolemmal Ca^{2+}-ATPase (Eisner et al., 2000). Studies have shown that most of the cytosolic Ca^{2+} rise during ischemia is mainly due to Ca^{2+} entry by reverse-mode, which occurs secondary to the rise in Na^{+} during ischemia (Imahashi et al., 2005). This occurs due to the increased generation of protons during ischemia, which are forced out from the cell via Na^{+}-

H^+ exchange (NHE), leading to a rise in intracellular Na^+ (Murphy et al., 1991). The increase in intracellular Na^+ will result in a reversal of (NCX) to bring more Ca^{2+} into the myocyte.

In a previous animal study, NHE inhibitors such as cariporide show reduction in the rise in Na^+ and to reduce ischemia-reperfusion injury especially when administered before ischemia (Murphy et al. 1991). On reperfusion of myocardium, there is a sudden increase in intracellular Ca^{2+}, which is due to the damage of sarcolemmal-membrane and oxidative stress which induces impairment of the sarcoplasmic reticulum function. These two forms of injury will cause imbalance of the normal mechanisms that regulate Ca^{2+} in the cardiomyocyte; this phenomenon is called the calcium paradox (Piper et al. 1998). This will lead to an increase in the mitochondrial Ca^{2+} and intracellular Ca^{2+}, which will induce cardiomyocyte death due to hypercontracture of heart cells and mitochondrial permeability transition pore (MPTP) opening (Piper et al., 1998)

Hypercontracture occurs after a period of ischemia, where cardiomyocytes may react to re-energization by developing a sudden and severe distortion of their architecture, which result from the excessive contractile force (Ganote et al., 1983). This hypercontacture phenomenon will lead to excessive cell shortening and may occur during the first few minutes of reperfusion as a result of adenosine triphosphate (ATP) availability in the presence of high Ca^{2+} concentration and cytoskeletal fragility that occurred due to ischemia (Ladilov et al., 1997).

2.1.2. Mitochondria in IRI

Mitochondria play a crucial role in cell survival and death (Dawson et al., 1993). The regulation of mitochondrial antioxidant status and the inhibition of mitochondrial permeability transition (MPT) pore formation can increase the protection of the myocardium to ischemia – reperfusion induced damage (Chiu et al., 2007).

The mitochondrial permeability transition (MPT) pore is a protein pore that is found in the mitochondrial inner membrane. It was originally discovered by Haworth and Hunter in 1979, where they found that induction of the permeability transition pore can lead to mitochondrial swelling and cell death. MPT was found to play a major role in cell damage caused by ischemia. However, research has shown that during ischemia, the MPT pore remains closed, but during reperfusion, the MPT pore opens (Bopassa et al., 2005).

Crompton et al. (1987) were the first to discover the crucial role of MPT in cardiac ischemia-reperfusion injury. It was confirmed that during ischemia, MPT is inhibited by several mechanisms which include acidosis, increased Mg^{2+}, elevated cytosolic Ca^{2+} and minimal reactive oxygen species (ROS) production (Fig. 2.1), while during reperfusion, after re-oxygenation and resumption of electron transport, an excessive production of ROS is induced at the same time when phosphate and intracellular Ca^{2+} are increased and intracellular Mg^{2+} is decreased (Weiss et al., 2003). All these factors that occur during reperfusion promote MPT pore opening, which will result in myocardial damage during ischemia-reperfusion injury.

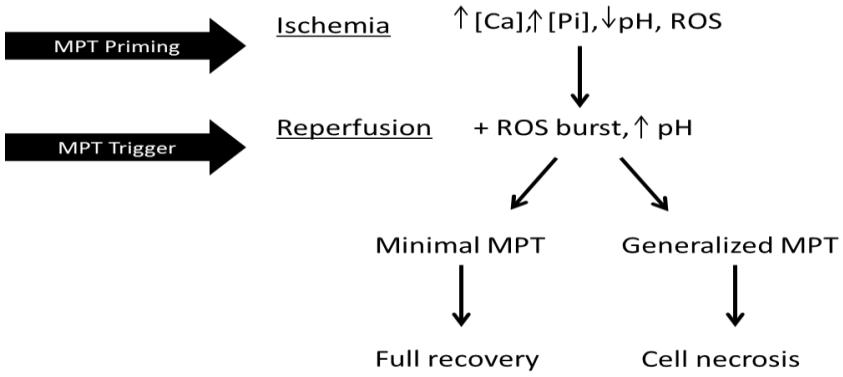


Fig 2.1. MPT and cell fate during ischemia/reperfusion. Hypothetical role of MPT in cell death through apoptosis during ischemia reperfusion of the heart. Pi indicates inorganic phosphate; ROS, reactive oxygen species (Modified from Weiss et al., 2003).

2.1.3. Reactive oxygen species (ROS) in IRI

Reactive oxygen species (ROS) are a number of reactive molecules and free radicals derived from molecular oxygen (Zucchi et al., 2007). Due to their high reactivity, they can start reactions that lead to irreversible changes in lipids and proteins (Zucchi et al., 2007). During several physiological conditions, aerobic cells produce ROS, but due to the existence of endogenous scavenger systems, the cellular macromolecules are protected against reactive oxygen species induced injury (Hallowell et al., 1999).

The main chemical species in ROS are superoxide radical (O_2^-), hydroxyl radical ($^{\bullet}HO$), hydrogen peroxide (H_2O_2) and peroxynitrate ($ONOO^-$) (Halliwell et al., 1999). All aerobic cells contain scavenging enzymes that are able to protect against ROS. The most important enzymes are glutathione peroxidase, superoxide dismutase (SOD) and catalase (Mao et al., 1993). In several conditions, ROS generation can overwhelm the capacity of the cellular scavenging enzymes, which is a condition called oxidative stress (Giordano et al., 2005). Oxidative stress is well known for its pathophysiological role in ischemia-reperfusion injury (Dhalla et al., 2000).

The pathophysiological role of ROS in myocardial ischemia-reperfusion has been suggested as a result of the increase in myocardial ROS concentration after ischemia and during the first few minutes of reperfusion (Kramer et al., 1987) and ROS species have also been recognized in the reperfused myocardium (Giordano el al., 2005).

In the condition of myocardial ischemia-reperfusion injury, several studies have shown the biochemical evidence of ROS induced injury in which there was a remarkable increase in tissue concentrations of malondialdehyde, which confirm the evidence of occurrence of lipid peroxidation (Dudda et al., 1996; Tavazzi et al., 1998).

Mitochondria are a major source of ROS production via the respiratory chain especially H_2O_2 production, which increases remarkably during early reperfusion, possibly as a result to the modification in the phospholipid environment and the damage of electron transport between complex I and complex III (Turrens et al., 1991; Paradies et al., 2004). Furthermore, there is evidence that cardiomyocyte NADPH oxidases are the major source of ROS (Xiao et al., 2002; Soccio et al., 2005). Inhibition of electron transport at complex I and mitochondrial uncouplers have shown to protect against ischemia-reperfusion injury, it was proposed that this protection was due to the reduction of ROS production during ischemia and reperfusion (Lesnefsky et al., 2004; Sack et al., 2006). Reactive oxygen species are also thought to be involved in the dysfunction of critical proteins which include the Na^+-Ca^{2+} exchanger, cytochrome P450, sarcolemmal and sarcoplasmic reticulum Ca^{2+} channels (Rashar et al., 2008; Zucchi et al., 1995).

2.2.1. Role of Ascorbic acid in IRI

Ascorbic acid is well known for its antioxidant activity, because as a reducing agent it can reduce and neutralize reactive oxygen species (Padayatty et al., 2003). However, ascorbic acid not only behaves as an antioxidant, but sometimes also as a pro-oxidant (McGregor et al., 2006). Ascorbic acid has been shown to reduce transition metals, such as cupric ions (Cu^{2+}) to cuprous (Cu^+), and ferric ions (Fe^{3+}) to ferrous (Fe^{2+}) during conversion from ascorbate to dehydroascorbate in vitro (Satoh et al., 1997). This reaction can generate superoxide and other reactive oxygen species. However, in the body, free transition metal ions are unlikely to be present while iron and copper ions are bound to diverse proteins (McGregor et al., 2006) and the intravenous use of vitamin C does not appear to increase pro-oxidant activity under normal conditions (Muhlhofer et al., 2004). But in ischemia-reperfusion, metals may become decompartmentalized, and in these conditions ascorbic acid may act as a pro-oxidant.

Although previous studies on vitamin C supplementation in human cardiovascular diseases either alone or in combination with other antioxidants such as vitamin E did not prove great benefits clinically and in some cases even showed some harm (Sesso et al., 2008), vitamin C may still play a role in decreasing cardiovascular diseases (Bazzano et al., 2003).

One study in an animal model of IRI found that ascorbate and glutathione are the first antioxidants to be depleted in heart tissues, followed by vitamin E and ubiquinone (Haramaki et al., 1998). However, the protective effects of ascorbate specifically to myocardial ischemia-reperfusion injury have shown inconsistent and conflicting results. In some previous studies, delivery of 10-100 μM ascorbate in the perfusate after ischemia did not provide protection to isolated rabbit hearts against I/R injury (Holdefer et al., 1994; Rump et al., 1998); contrarily, detrimental effects of ascorbate have also been reported when added to rat heart perfusate (van Jaarsveld et al., 1994) or when delivered intravenously to rabbits (Tsovolos et al., 2008) or humans (Bailey et al., 2006) prior to surgical interventions involving ischemia-reperfusion. The detrimental effect was suggested to be mediated by iron-catalyzed oxidation of ascorbate, with consequent reactive oxygen species generation (Bailey et al., 2006).

Several other studies in isolated rat hearts have shown protective effects of ascorbate. For example, addition of 1 or 10 mM ascorbate to the perfusate prior to global ischemia and reperfusion improved recovery of aortic flow (Chambers et al., 1989). Furthermore, vitamin C (100 μM) in the perfusate at the time of reperfusion showed protection against microvascular structural damage, which occurs in the reperfused myocardium (Molyneux et al., 2002). Gao et al. (2002) showed that the addition of vitamin C (1 mM) to the perfusate of isolated rat hearts at the time of reperfusion had no significant benefits alone, but it improved protection by 1 mM glutathione monoethyl ester.

In studies of oral delivery of ascorbate, several have shown protection against IR injury. In one study of cardiac surgeries in dogs and humans, prior and post-delivery of ascorbate orally or intravenously (in dogs) resulted in a decrease of post-operative fibrillations and oxidative stress (Carnes et al., 2001). In a clinical study of blood oxidative parameters in post myocardial infarction thrombolysis patients, vitamin C supplementation showed a significant increase of blood superoxide dismutase (SOD) activity, decrease of xanthine oxidase activity and tremendous decrease of malondialdehyde (MDA) levels (Bhakuni et al., 2006).

In a study with rats, feeding vitamin C for seven days increased vitamin C content of the heart and lowered some of the parameters of oxidative stress during subsequent IRI of isolated hearts (Sicard et al., 2006). Kearns et al., (2001) demonstrated that prior short term oral therapy with vitamin C attenuated reperfusion- induced injury in skeletal muscle of rats by antioxidant effects and an anti-inflammatory effect by attenuating neutrophil respiratory burst activity. In such dietary studies of vitamin C, the diet would usually also contain flavonoids, which may facilitate vitamin C actions. However no studies have examined a potential synergistic or cooperative effect of ascorbate and flavonoids in IR injury.

A previous in vitro study (Bandy and Bechara, 2001) indicated that certain flavonoids may have a synergistic interaction with ascorbate that could provide potent protection to membranes such as those of mitochondria. Since mitochondria play an important role in ischemia-reperfusion and cell death, this interaction may be important in protecting the heart from I/R injury. Flavonoids that were effective in this cooperation in vitro were quercetin and a catechin monomer such as epicatechin (Bandy and Bechara, 2001).

2.2.2. Glutathione

Glutathione (GSH) is the main low-molecular-weight thiol in all aerobic cells. It is formed in the body from three amino acids, L-glutamic acid, glycine and L-cysteine. Glutathione or γ-glutamyl-cysteinyl-glycine is found in cells in millimolar (0.5-10) concentrations (Lash et al., 2006). Its effect is mainly due to the presence of the thiol group on the cysteinyl residue; where the thiol enables GSH to function as both a reductant and a nucleophile. Glutathione is mainly synthesized in the liver, most of cellular glutathione (85-90%) is found in the cytosol with the remainder in mitochondria and nuclear matrix (Lu et al., 2000). Glutathione plays a major role in many cellular reactions; the most important is scavenging free radicals and reactive oxygen species such as hydroxyl radical, peroxynitrite and hydrogen peroxide through enzymatic reactions (Fang et al., 2002).

Glutathione disulfide (GSSG) is the oxidized form of glutathione and intracellular glutathione is adequately maintained in the reduced state by glutathione reductase. On exposure to oxidative stress, the ratio of GSH/GSSG will be reduced due to accumulation of GSSG. Thereby, determination of GSH/GSSG ratio is a valuable indicator of oxidative stress (Akerboom et al., 1981).

Singh et al. (1989) reported that glutathione levels in the heart tissue following ischemia-reperfusion could affect the intensity of myocardial damage. In a previous study, glutathione has reduced the post-ischemic injury in rat hearts especially after the addition of ascorbate which implies the possible synergism between the two hydrophilic antioxidants (Gao et al. 2002).

2.3. Mechanisms of flavonoid protection against IRI

Flavonoids are a subgroup of the polyphenol family. There are more than five thousand flavonoids that have been identified, and they share a basic structure containing two benzene rings with a pyran ring in the middle (Fig 2.2) (Ross et al., 2002). Albert Szent- Györgyi (1936) was the first to discover flavonoids, and referred to this class of compounds as vitamin P. Dr. Szent-Györgyi found that vitamin C was not as effective as when vitamin P (flavonoids) was added to the mixture which led to improvement of the blood vessel capillaries.

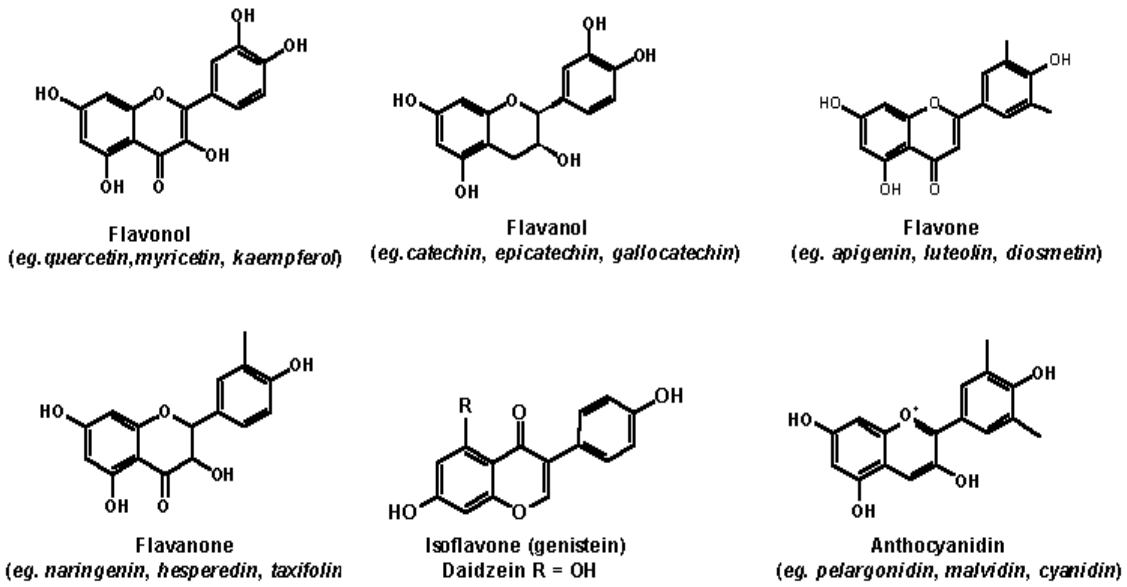


Fig 2.2. Different classes of flavonoids

Flavonoids are known for their antioxidant activity and as they are found extensively in fruits and vegetables. They may be involved in the currently known health benefits of plant foods (Arts et al., 2005).

In several studies, flavonoids have shown promise in preventing ischemia-reperfusion injury through different mechanisms, which include antioxidation through free radical scavenging (Schewe et al., 2008), anti-inflammation (Youdim et al., 2002) and vasodilation (Bell et al., 2006). Recently, it was suggested that flavonoids might affect endogenous antioxidant defenses of cells by modulating glutathione-related enzyme systems and glutathione (Masella et al., 2005).

Although previous studies revealed that antioxidants such as vitamin C or vitamin E can help to partially protect against reperfusion injury in some model systems, they did not always have clinical success in protecting the heart from ischemia-reperfusion injury (Dhalla et al., 2000). The different mechanisms of flavonoid protection against myocardial ischemia-reperfusion injury were reviewed previously (Akhlaghi and Bandy, 2009) and will be highlighted in the following sections.

2.3.1. Scavenging of reactive oxygen species

Flavonoids are hypothesized to protect against myocardial ischemia-reperfusion injury by scavenging ROS, since they are potent scavengers of reactive species such as peroxyl radicals (Nakao et al., 1998), superoxide (Chun et al., 2003) and peroxynitrite (Pollard et al., 2006). In a previous study, after 3 weeks feeding with proanthocyanidins, there was a decrease in the electron spin resonance, which detects the generation of free radicals during the first few minutes of reperfusion (Pataki et al., 2002). Furthermore, oral treatment with quercetin for one week before the induction of ischemia has been shown to decrease malondialdehyde levels in heart

tissue, which is a marker for oxidative damage (Ikizler et al., 2007). Moreover, scavenging of ROS showed other benefits as a result of scavenging superoxide radicals, the bioavailability of nitric oxide (NO) increases (Huk et al., 1998; Freedman et al., 2001) which is expected to improve the endothelial function in the post-ischemic heart.

2.3.2. Metal chelation

It has been shown that some of the antioxidant effects of flavonoids are delivered through chelation of metal ions such as copper and iron (Morel et al., 1993; Maffei et al., 1996). Since transition metal ions are major co-factors for the Fenton reaction, their chelation by flavonoids can prevent their unwanted effects resulting from this kind of reaction (Gua et al., 2007; Cheng et al., 2000). Decompartmentalization of iron has been found to be involved in the process of oxidative stress in myocardial ischemia-reperfusion injury (Berenshtein et al., 1997; Horwitz et al., 1999), but this iron induced damage was found to be inhibited by perfusing the heart with the flavonoid catechin (Voogd et al., 1992). Interestingly, it has been proposed that when specific flavonoids bind to metals, they may act similar to superoxide dismutase, scavenging superoxide more potently and inhibiting the catalytic activity for the Fenton conversion of hydrogen peroxide to hydroxyl radicals (Kostyuk et al., 2007; Malesev et al., 2007).

2.3.3. Inhibition of xanthine oxidase

Xanthine oxidase is a form of xanthine oxidoreductase, which is an enzyme that was found to generate reactive oxygen species such as hydrogen peroxide and superoxide radicals (Taras et al., 2004). Flavonoids may inhibit xanthine oxidase, which may be one of their mechanisms in reducing ischemia-reperfusion injury. Several flavonoids including quercetin, apigenin and luteolin have shown to inhibit xanthine oxidase (Cos et al., 1998; Lin et al., 2002). In a study by Ashraf et al. (1993), it was verified that xanthine oxidase activity occurs in coronary vessels and

interstitial cells, so it possibly plays a role in ischemia-reperfusion injury. This finding led to the notion that inhibiting xanthine oxidase may have a protective effect against ischemia-reperfusion injury.

2.3.4. Reinforcement of cellular antioxidants

Antioxidants can work in a network by scavenging different reactive species and interacting with each other (Packer et al., 1995). For example, ascorbate can react with aqueous radicals such as $O_2^{\bullet -}$ while vitamin E acts in membranes where it scavenges lipid peroxyl radicals. Vitamin E is suggested to be regenerated from its radical at the surface of membranes by the effect of ascorbate, while ascorbate alternatively is regenerated by glutathione (Fig. 2.3).

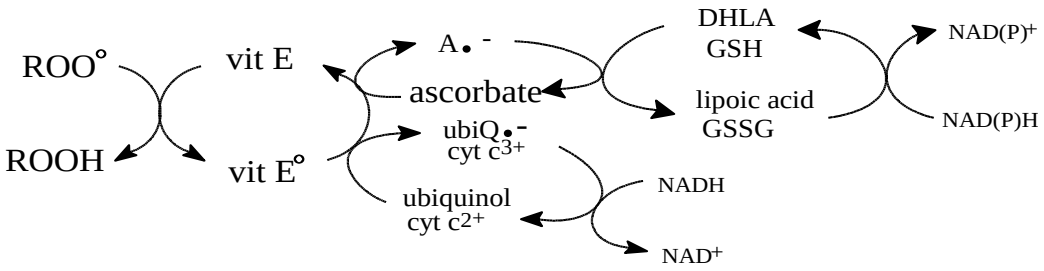


Fig 2.3. Antioxidant interactions in peroxide-induced stress to mitochondria. Adapted from (Packer et al., 1995).

In myocardial ischemia-reperfusion injury, human studies have shown that there is a depletion of non-enzymatic antioxidants such as ascorbic acid and vitamin E (Marczin et al., 2003). Interestingly, some hydrophilic antioxidants such as ascorbate and glutathione have been shown to be the first line of defense against oxidative stress, as they protected lipophilic antioxidants such as vitamin E from oxidation (Haramaki et al., 1998). In vitro, ascorbic acid is shown to help vitamin E regeneration from its oxidized form (Nagaoka et al., 2007).

This protective network proposed that flavonoids can act as intermediate antioxidants, where they protect lipophilic antioxidants and at the same time are being protected by hydrophilic antioxidants (Lotito et al., 1999; Lotito et al., 2000). Although this seems to have promising results, the extent to which flavonoids may cooperate to protect other antioxidants in myocardial ischemia–reperfusion injury has not yet been studied.

2.3.5. Inhibition of metalloproteinases

Matrix metalloprotinases (MMP) are a family of proteases, which play an important role in protein degradation and tissue remodeling (Spinale et al., 2007). Several studies have verified a direct relationship between the formation of reactive oxygen species (ROS) and the induction of MMP during ischemia reperfusion injury (Joffs et al., 2001; Lin et al., 2005). Additionally, in vitro studies have also shown that direct exposure to ROS will induce the release of MMP (Belkhiri et al., 1997; Cheung et al., 2000). Studies have shown that the plasma levels of MMP increase after ischemia reperfusion related morbidities such as myocardial infarction (Phatharajaree et al., 2007) and heart failure (Yan et al., 2006). Since increased activity of MMP is involved in ventricular dilatation and cardiac remodeling (Janicki et al., 2004), inhibitors of MMP may play a major protective role to prevent the complications of the ischemia reperfusion injury (Sang et al., 2006).

Flavonoids have shown promising results by inhibiting the activation of metalloproteinase-2 (Oak et al., 2005). In an earlier study, which involved human umbilical endothelial cells, catechin reduced the activation of metalloproteinase-2 (Oku et al., 2003). Moreover, it has been shown that quercetin has shown the ability to decrease the expression of metalloproteinase-9 in human aortic smooth muscle cells (Moon et al., 2003).

2.3.6. Vasorelaxation effects

In addition to their antioxidant activities, flavonoids such as resveratrol and quercetin have also been shown to have a vasodilator effect (Chen et al., 1996), which may help in reducing ischemia reperfusion injury. Nitric oxide (NO) is a signaling molecule with vasodilator and anti-inflammatory activities (Laursen et al., 2006; Reichenbach et al., 2005). Flavonoids are shown to increase endothelial nitric oxide synthase (eNOS) activity (Benito et al. 2002) and also induce eNOS expression (Olszanecki et al. 2002; Hung et al. 2004). Hotta et al. (2006) and Potenza et al. (2007) both tested the effect of epigallocatechin gallate in an ischemic reperfused heart and concluded that the induction of eNOS was part of the beneficial effect that was provided by epigallocatechin gallate Another mechanism by which flavonoids can produce vasodilation is by scavenging superoxide, which otherwise reacts rapidly with nitric oxide (producing peroxynitrite) and prevents its vasodilator effect. Flavonoids may also provide vasodilatation by stimulating endothelial cells to produce prostacyclin which is an effective vasodilator (Maffei et al., 1999). Oral administrations of grape seed proanthocyanidins for three weeks have been shown to increase the production of prostacyclin in ischemic reperfused heart (Maffei et al., 1999).

2.3.7. Anti-inflammatory effects

The effect of flavonoids on endothelial tissue and their ability to reduce inflammation was reported in 1936 by Szent-Györgyi and Ruszenyak. These two researchers discovered the effect of a substance from lemon (citrin), which improved the capillary resistance of vascular purpura patients (Szent-Györgyi et al., 1936). Several mechanisms that have been described have shown the anti-inflammatory activity of flavonoids, including anti-oxidative activities, modulation of cellular activities of inflammation-related cells such as macrophages, mast cells

and neutrophils (Middleton et al., 2000), regulation of the production of other proinflammatory molecules and modulation of proinflammatory gene expression. Also, certain flavonoids have shown the ability to modulate phospholipase A_2 (PLA_2) (Chang et al., 1994), lipoxygenase (LOX) and cyclooxygenase (COX) (Chi et al., 2001), which are metabolizing enzymes of arachidonic acid (AA). A suppression of these enzymes will decrease the production of crucial mediators of inflammation such as leukotrienes (LT) and prostaglandins (PG) (Kim et al., 2004).

In a previous study, catechins and quercetin have shown their inhibitory action on IL-1β and TNFα to improve the release of the anti-inflammatory cytokine IL-10 (Santangelo et al., 2007). Although some studies have shown that flavonoids potentially have anti-inflammatory action, the effect in human subjects is still unclear.

2.4. Previous studies on catechins in IRI

Among flavonoids, the catechins have gained special attention as potential cardioprotective agents (Islam et al., 2012). Catechins are flavonoids richly present in teas and cocoa, with green tea being especially rich in bioavailable catechin monomers. The term catechin is commonly used to refer to the related family of flavonoids and the sub group flavan-3-ols (or simply flavanols). The name of the catechin chemical family derives from catechu, which is the juice or boiled extract of mimosa catechu (Acacia catechu L.F) (Zheng et al., 2008). The major catechins of green tea are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin-3 gallate (EGCG) (Balentine et al., 1997; Dufresne et al., 2001; Higdon et al., 2003). Catechins exert vascular protective effects through multiple mechanisms, including antioxidant, antihypertensive, anti-inflammatory, anti- proliferative, anti- thrombogenic and lipid lowering effects (Balentine et al., 1997).

Catechins antioxidant activity is due to scavenging of free radicals, chelating redox active transition-metal ions, decreasing redox- active transcription factors, inhibiting pro-oxidant enzymes and inducing antioxidant enzymes (Dufresne et al., 2001). Tea catechins also decrease the key enzymes involved in lipid biosynthesis and reduce intestinal lipid absorption, thereby improving blood lipid profile (Higdon et al., 2003). Experimental and clinical studies suggest that tea catechins can significantly improve endothelial function, thereby providing additional beneficial effect in patients with CVD (Potenza et al., 2007).

Catechins can also help in the prevention of oxidative stress-induced apoptosis (Kumar et al., 2002) and can inhibit p38 MAPK phosphorylation and subsequently reduce apoptosis (Townsend et al., 2004). Several studies have shown that reduction of P38 MAPK phosphorylation leads to reduction of apoptosis and improvement of cardiac function following myocardial ischemia-reperfusion injury (Ma et al., 1999).

Catechins appear to be a powerful cardioprotective group of flavonoids that have shown promising results before or during ischemia-reperfusion in the heart, including limitation of infarction size and reduction of apoptosis when delivered in vitro or in vivo (Potenza et al., 2007; Townsend et al., 2004; Yamazaki et al., 2008; Modun et al., 2003; Aneja et al., 2004; van Jaarsveld et al., 1996). Furthermore, feeding green tea catechins to rats for 10 days has been shown to have a protective effect in isolated hearts against ischemia-reperfusion injury and increased the phase 2 enzyme levels (Akhlaghi and Bandy, 2010). In other studies, oral delivery of catechins assisted in the left ventricular function recovery after reperfusion (Yanagi et al., 2011), and dietary catechin showed significant reduction of oxidative stress in the reperfusion injury, indicated by decreasing the levels of MDA (Kumar et al., 2002).

Previous in vitro studies on protection by catechins against IR injury have used very high levels of catechins (10-100 µM), and no studies have looked at the combination of a catechin and ascorbate in protecting from myocardial ischemia-reperfusion injury. In in vitro experiments, epicatechin was identified as a flavonoid which might be able to cooperate with ascorbate in protecting membranes and mitochondria against oxidative damage (Bandy and Bechara, 2001).

2.5. Possible cooperation of ascorbate and flavonoids in IRI

In 1936, Albert Szent- Györgyi and coworkers (Bensath et al., 1936, 1937; Rusznyak and Szent- Györgyi, 1936) found a vitamin like principle in lime juice that they referred to as flavone or flavonol glucoside, which they called "citrin". They discovered that citrin is able to treat some of the capillary fragility complications that occur in humans, whereas vitamin C alone did not produce the same effect; additionally they also found that 1mg/day of citrin could extend the life of vitamin C deficient guinea pigs from 28.5 to 44 days. This led to the suggestion that citrin is able to stabilize and potentiate the biological activity of ascorbate (Ruszenyak and Szent-Györgyi, 1936; Bensath et al. 1937). Antioxidants can act in different ways; either by acting in the aqueous phase to react with aqueous radicals, such as with ascorbate, or acting in membranes to scavenge lipid peroxyl radicals, such as with vitamin E (Packer et al., 1995).

In a previous study (Bandy and Bechara, 2001), some flavonoids were shown to transfer electrons from ascorbate to a hydrophobic environment and therefore they mediate antioxidant protection from ascorbate to membrane-bound cytochrome c in its reaction with peroxides. This protection is important because it has been suggested that the peroxidatic activity of cytochrome c may be involved in its release from the mitochondrial inner membrane in the trigger for apoptosis of cells undergoing oxidative stress (Davison et al., 2002; Kagan et al., 2004). These results suggest that during cardiac ischemia-reperfusion injury where oxidative stress occurs, a

combination of flavonoids and ascorbate may be synergistically beneficial in protecting against apoptosis. Previous studies have looked at protection against IRI by ascorbate alone or flavonoids alone usually at high concentrations; however no studies have looked at the possible synergism of different flavonoids and ascorbate in protecting from myocardial ischemia-reperfusion injury.

2.6. Ischemia-Reperfusion injury models

The two major categories of experimental myocardial ischemia-reperfusion injury in an isolated rat heart are global ischemia and focal ischemia. Global ischemia is when the coronary blood flow is reduced throughout most or all of the heart tissue, whereas, focal ischemia is defined as a reduction in blood flow to a specific myocardial region. In global ischemia, blood flow ceases completely to the whole heart while in focal ischemia there is no blood flow in the central core of the ischemia but there is some flow that reaches other areas via collaterals. Global ischemia (or no-flow ischemia) can be achieved by interrupting the coronary arteries by either stopping the inflow lines or partially restricting the perfusate flow rate, followed by circulation restoration (reperfusion). This type of ischemia might be seen during open heart surgery when aortic cross clamping is required, and in heart transplantation. On the other hand, focal ischemia can be achieved by ligating a coronary artery; the most frequently used artery is the left ascending coronary artery (Valentin et al., 2004; Ytrehus et al., 2000). Coronary artery ligation can be done in vivo during open heart surgery, or ex vivo with an isolated perfused heart. In our study, a global ischemia model (Langendorff heart) was used due to the simplicity of the preparation, low cost and the ability to test our research hypothesis clearly.

The Langendorff working heart was established in 1895 by the German physiologist Oskar Langendorff as a tool for studying heart physiology (Zimmer et al., 1998). Briefly, the main

principle of the technique is to keep cardiac activity by perfusing the heart through the coronary arteries using an aortic cannula where the ascending aorta is inserted; through this cannula, the perfusion solution is delivered to the heart in a retrograde manner. The retrograde perfusion causes the closure of the aortic valve and deviates the entire perfusate flow through the coronary ostia into the coronary arteries; the perfusate passes through the coronary circulation and drains into the right atrium via the coronary sinus and is driven out through the right ventricle. Although, the isolated perfused heart technique has some limitations such as the absence of the normal humoral effects of the heart, the high perfusion pressure of the retrograde flow in the aorta that may lead to incompetence of the aortic valve causing complete blockage of the entire perfusate through the coronary circulation and the surgical preparation of the heart which requires a certain degree of skills because the heart is susceptible to contusion injuries, but until today the technique has been actively used as a valuable method in cardiovascular research. However, the durations of ischemia and reperfusion vary, and most studies do not compare with sham and continuous-perfusion controls (Modun et al., 2003; Korkmaz et al., 2009) to establish the extent to which damage is due to ischemia and reperfusion versus heart isolation and ex vivo perfusion. This study therefore compared the IR model with both of these controls.

2.7. Hypothesis

We hypothesized that vitamin C can act synergistically with catechin to protect the myocardium from ischemia-reperfusion injury.

2.8. Objectives

1. To determine the extent to which ascorbate or catechin alone, at levels which could be in blood after dietary supplementation, can protect myocardial tissue in the situation of ischemia-reperfusion injury.
2. To evaluate the effect of vitamin C and catechin when given together and the possible synergistic protective effect to the rat heart against ischemia-reperfusion injury.
3. To evaluate the strength of the IR model using sham and continuous-perfusion controls.

CHAPTER 3: MATERIALS AND METHODS

3.1 Animals and experimental groups

Animals were male Wistar rats weighing 200-250g. All animals were maintained and handled in compliance with the Canadian Council on Animal Care (CCAC) guidelines and with the approval of the University Committee on Animal Care and Supply (UCACS). The animals were assigned into six experimental groups (Fig. 3.1):

1) Sham group (n=8). Hearts were exposed to 15 min stabilization only.
2) Perfusion Control group (n=8). Hearts were perfused without any induction of ischemia for 135 min.
3) Ischemia-reperfusion group (n=8). Hearts were exposed to 30 min global ischemia and 90 min reperfusion.
4) Ischemia-reperfusion plus vitamin C group (n=8). Hearts were exposed to 30 min global ischemia and 90 min reperfusion, with 150 µM ascorbate in the reperfusion buffer.
5) Ischemia-reperfusion plus catechin group (n=8). Hearts were exposed to 30 min global ischemia and 90 min reperfusion, with 5 µM catechin in the reperfusion buffer.
6) Ischemia-reperfusion plus vitamin C and catechin group (n=8). Hearts were exposed to 30 min global ischemia and 90 min reperfusion, with 150 µM ascorbate and 5 µM catechin in the reperfusion buffer.

(+)- Catechin hydrate 98% and L-ascorbic acid 98% were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). L-ascorbic acid (150 µM) and catechin (5 µM) were prepared by dissolving the powder directly in the perfusate used for reperfusion after the induction of ischemia. These doses represent the maximum that might be obtained in the blood

with dietary supplementation, and based on the literature (Bartekova et al., 2010; Scarabelli et al., 2009) this dose of flavonoid alone may give mild protection.

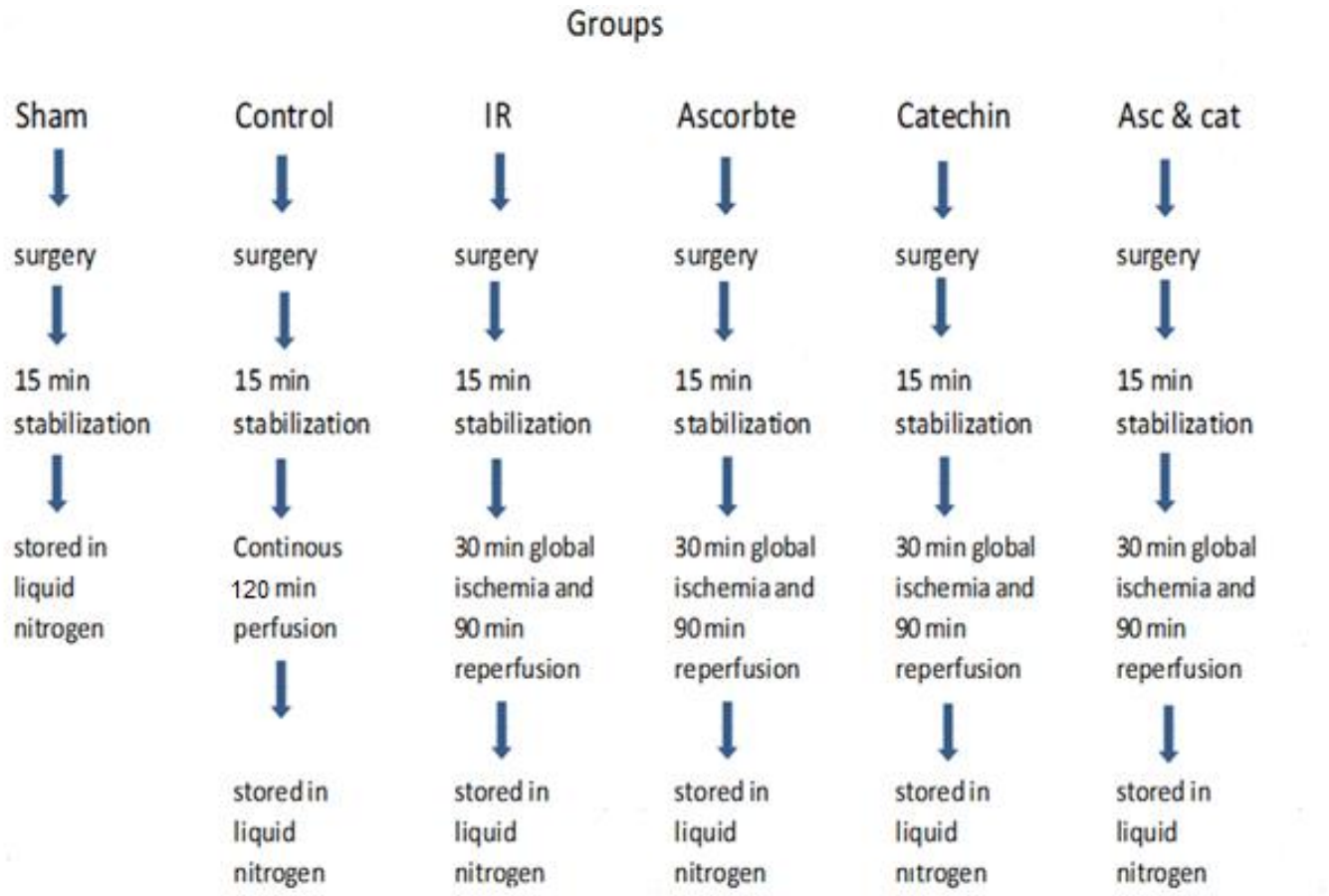


Figure 3.1 Consort diagram of the experiment.

3.2 Ischemia-reperfusion

The animals were anaesthetized with an initial flow of 5% isoflurane and maintenance flow of 3% in 1L/min of 100% oxygen. The animals were heparinized via intracardiac injection with 350 IU/Kg heparin. Then the chest wall was fully opened and the heart was removed, the heart was immediately immersed in cold perfusate and cannulated through the aorta on a Langerndorff apparatus. The maximum time between removal of the heart and cannulation through the aorta was six minutes; any heart that exceeded this time period was excluded from the results.

The hearts were perfused in the retrograde mode with modified Krebs-Henseleit buffer which contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 11mM glucose, pH 7.4 (Imahashi et al., 1999). The perfusate was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The perfusate was warmed to 37^0C using a heating coil connected to a circulating water bath and the temperature of the heart was maintained at 37^0C by the warm buffer in addition to a small water-jacketed chamber.

The sham group was used as a surgical control group in our experiment, which is commonly used in any study involving a surgical procedure to assure that the scientific data indicate the effects of the experiment itself and not the consequence of the surgery. The perfusion control group in our study was designed to keep the heart perfused for the entire time without any induction of ischemia.

All the hearts were exposed to a 15 min stabilization period before inducing ischemia. After the stabilization period, global ischemia was induced for 30 min by clamping the lower valve of the apparatus tube, and then the reperfusion was restored for 90 min by unclamping the lower valve rapidly. Ascorbate and catechin were added directly during the beginning of the

reperfusion period. At the end of reperfusion period, the hearts were snap-frozen in liquid nitrogen and kept at -70^{0}C for later analysis. The hearts from the sham group were frozen at the end of the 15 min stabilization period. The hearts from the perfusion control group were perfused for the stabilization period and continuously for a total of 135 min; while the hearts from the ischemia-reperfusion and the treated groups were exposed to ischemia for 30 min after the stabilization period and reperfusion for 90 min. The stabilization period was 15 minutes to ensure that contractile function and heart rhythm return to normal. The total number of rats was 48, but after excluding some hearts either due to the time between isolation of the heart and mounting the aorta exceeded 6 minutes, or due to abnormal heart rhythm after 15 minutes stabilization; the total number after exclusion was 39 rats as follows: sham group (n=6), control group (n=6), IR group (n=6), IR/ vitamin c group (n=7), IR/ catechin group (n=7), IR/ vitamin c/ catechin group (n=7).

3.3 Tissue crushing

Frozen hearts were crushed to a powder using a tissue pulverizer which was prechilled in liquid nitrogen. For tissue analysis, samples of pulverized frozen tissue were weighed out and homogenized in the appropriate buffer using a microcentrifuge tube homogenizer.

3.4 Caspase-3 activity

Caspase-3 activity was determined by incubation of tissue homogenates with a caspase-3 substrate and colorimetric detection of the cleaved substrate due to digestion by caspase-3 in cell lysate (Masini et al., 2003). Briefly, 0.03 g of the frozen tissue was homogenized in 500 µl cold lysis buffer containing 50 mM KCl, 10 mM Hepes, 5 mM MgCl, 10 mM dithiothreitol (DTT),

1mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, 2 µg/ml aprotinin and 0.5% CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1- propanesulfonate), pH 7.4. The homogenated tissues were incubated on ice for 15 min and centrifuged at 10,000 *g* for 10 min at 4°C. Then 6 µl of 10 mM caspase-3 substrate Ac-DEVD-p-nitroanilide (EMD Biosciences, San Diego, CA, USA) was incubated with 170 µl of the sample supernatant for 1h at 37°C, and the absorbance of the cleaved substrate p-nitroanilide, was measured at 405 nm. The extinction coefficient of 8300 M^{-1} cm^{-1} was used to determine the p-nitroanilide concentration. The activity was calculated as µmol of p-nitroanilide/min/mg of protein.

3.5 Thiobarbituric acid reactive substances (TBARS)

Peroxidation in tissues was detected by the TBARS assay based on the reaction of thiobarbituric acid with reactive aldehydes present in the tissue samples, forming a colorimetric adduct that could be detected using spectrophotometer (Ohkawa et al. 1979). Briefly, 0.05 g of the frozen tissues was homogenized with 200 µl of either 3% metaphosphoric acid (MPA) or RIPA buffer containing 50 mM Tris, 150 mM KCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and 0.1% EDTA, pH 7.2. The homogenates were incubated for 30 min at room temperature, and centrifuged at 10,000 *g* for 10 min at 4°C. A 100 µl aliquot of homogenized tissue sample supernatant was added to 400 µl of thiobarbituric acid solution containing 0.02% BHT, 8.1% SDS, 0.8% thiobarbituric acid, and 20% acetic acid, pH 3.5, and heated for 1h at 95°C. The heated samples were cooled in cold water, centrifuged at 4,000 *g* for 10 min, and the absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated using a standard curve of malondialdehyde (MDA) prepared from tetraethoxypropane by acid hydrolysis (Esterbauer & Cheeseman, 1990).

3.6 Total malondialdehyde (MDA)

Lipid peroxidation is a well - established indicator of oxidative stress in tissues. In our study, we used the TBARS method to measure MDA in the samples. However, this reaction is non-specific to some extent; because other aldehydes and ketones can react. The MDA-586 method was designed to assay total MDA in the tissues (Gerrard- Monnier et al., 1997). Briefly, 0.06 g of the frozen tissue was homogenized with 300 µl diluted hydrochloric acid, pH 1.5. The homogenates were hydrolyzed for 80 minutes at 60°C and centrifuged at 2,000 *g* for 10 minutes at 4°C. A 100 µl aliquot of homogenized tissue sample supernatant was added to 325 µl of 15 mM 1-methyl-2-phenylindole and 75 µl of concentrated 37% hydrochloric acid. The resulting samples were incubated at 45°C for 40 minutes and centrifuged for 5 minutes at 2,000 *g*. The absorbance of the supernatant was measured at 586 nm. The concentration of malondialdehyde (MDA) was calculated using a standard curve of malondialdehyde prepared from 1, 1, 3, 3-tetraethoxypropane by acid hydrolysis (Esterbauer & Cheeseman, 1990).

3.7 Histological analysis using hematoxylin and eosin stain (H&E)

The hematoxylin and eosin (H&E) stain is considered the routinely used stain in histopathology which provides a detailed view of the tissue by staining cell structures including nucleus, cytoplasm and organelle components. Briefly, the heart was sliced into four 2 mm thickness cross-sectional pieces, all the slices were fixed in 10% formalin and were embedded in paraffin for 48 hours. Heart samples were sliced (5 µM) in the pathology lab (City Hospital, Saskatoon). The slides were assessed by a pathologist who was blinded to our experimental protocol. For the H&E stain, only one rat per group was used to detect if there was any degeneration, necrosis and edema.

3.8 Glutathione measurement

Tissue glutathione and glutathione disulfide were determined according to a method described by Akerboom and Sies (1981). Briefly, 0.05 g of tissue was mixed with 300 µl meta-phosphoric acid solution containing 3% meta-phosphoric acid. Homogenized samples were left on ice for 10 min and centrifuged at 10,000 *g* for 10 min at 4°C. For glutathione (GSH) measurements, 50 µl of the protein-precipitated supernatants was added to 140 µl of 50 mM potassium phosphate buffer, pH 8.5, mixed and the background absorbance was measured at 412 nm. Then, 2 µl of 10 mM 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) (in methanol) was added, mixed and incubated for 10 min in the dark. A second reading at 412 nm was performed and the difference between the two readings was used to determine the glutathione level in the tissue.

For glutathione disulfide (GSSG) measurement, 50 µl of the protein free supernatants was added to 144 µl of 50 mM potassium phosphate buffer, pH 8.5, with 4 µl of 10 mM NADPH. The initial absorbance was recorded at 340 nm, and 2 µl of (20 U/ml) glutathione reductase was added, mixed and the samples were incubated at room temperature for 40 min and read again. The differences of the two readings indicate NADPH consumption, and therefore the amount of glutathione disulfide present. Standard curves were plotted using pure glutathione disulfide and glutathione. The values for tissue glutathione were calculated according to the standard curves and weight of the tissues used.

3.9 Statistics

Statistical analysis was performed with Graph Pad Prism software using one-way analysis of variance (ANOVA). Data shown are means ± SEM. Comparisons were made between hearts in the different groups using 2-sided Tukey test as the post hoc test. A P value < 0.05 was considered significant.

CHAPTER 4: RESULTS

4.1 Glutathione and glutathione disulfide

Glutathione (GSH) is one of the most important endogenous antioxidants acting against reperfusion-induced oxidative stress, wherein it participates in the antioxidant network and may become oxidized. In our experiments using the isolated heart model, the level of heart GSH did not significantly decrease with ischemia-reperfusion compared to controls, and none of the treatments gave improvement of glutathione levels in the heart tissues (Fig 4.1). The level of glutathione disulfide (GSSG) in the hearts was also not significantly affected by ischemia-reperfusion or by any of the treatments, although there was a trend toward an increase in the ischemia-reperfusion group and a suggestion of protection by the treatments (Fig. 4.2). Similar to GSH and GSSG, there was no significant difference between groups in the GSH to GSSG ratio (Fig. 4.3).

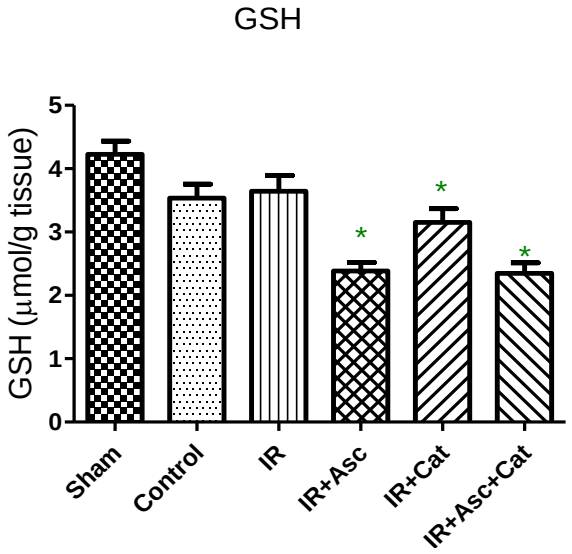


Figure 4.1 *The level of GSH in the heart tissue was determined with DTNB as described in materials and methods*. The bars show means ± SEM. * indicates significant difference between Sham, control, IR and treatments groups ($p < 0.05$) (ANOVA, p= 0.0001; Sham

vs IR+Asc, p= 0.0001; Sham vs IR+Cat, p= 0.0070; Sham vs IR+Asc+Cat, p= 0.0001; Control vs IR+Asc, p= 0.0033; Control vs IR+Asc+Cat, p= 0.0022, IR vs IR+Asc, p= 0.0011; IR vs IR+Asc+Cat,p= 0.0007).

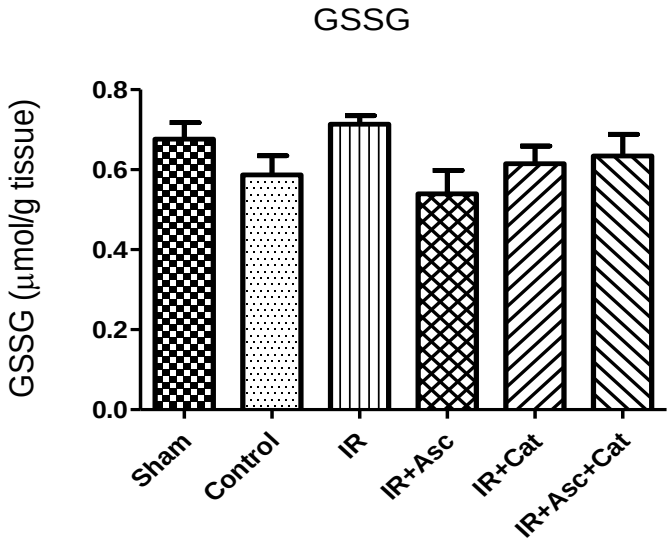


Figure 4.2 *The level of glutathione disulfide (GSSG) in the heart tissue was determined by oxidation of NADPH in the presence of glutathione reductase as described in materials and methods*. The bars show means ± SEM.

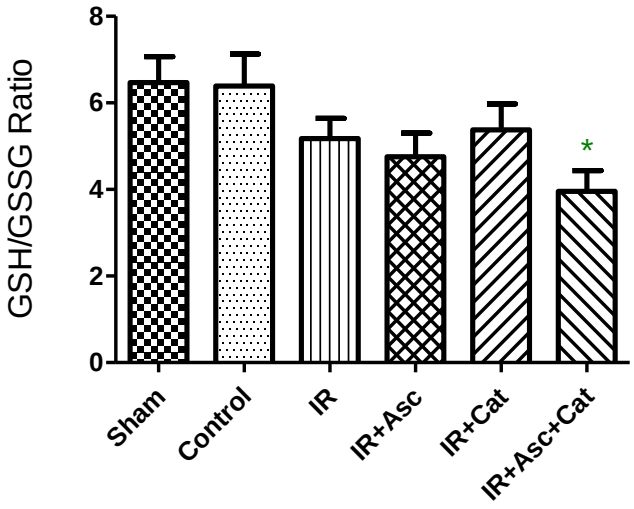


Figure 4.3 *Ratio of glutathione (GSH) to glutathione disulfide (GSSG) in heart tissues*.* indicates significantly different from Sham group ($p < 0.05$). (ANOVA, p= 0.0289; Sham vs IR+Asc+Cat, p= 0.0401).

4.2. TBARS

To assess ischemia-reperfusion induced oxidative stress in cardiac cells, TBARS which is an indicator of oxidative stress was measured using heart tissue homogenized in RIPA buffer (Fig 4.4) and MPA buffer (Fig 4.5). In assays using RIPA buffer (Fig 4.4), ascorbate, catechin and ascorbate plus catechin gave significant inhibition of lipid peroxidation compared to IR, which showed ≥ 100% protection relative to sham and control groups. None of the treatments gave statistically improvement when MPA buffer was used to homogenize the tissue (Fig 4.5), although the pattern was similar to that observed in RIPA buffer.

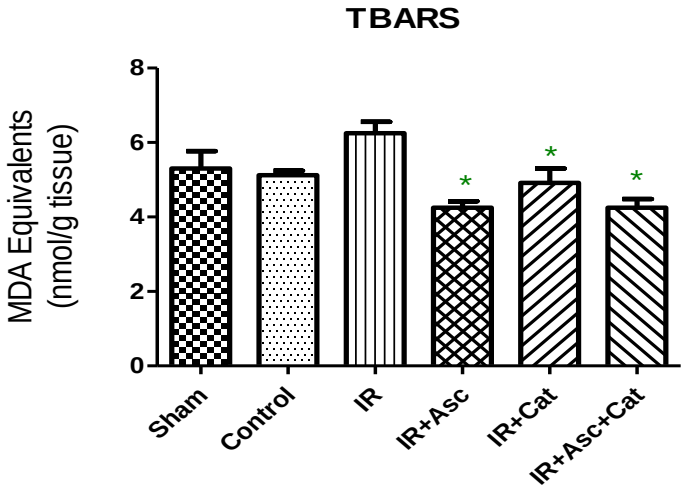


Figure 4.4 *Levels of TBARS in homogenates using RIPA buffer*. The TBARS assay was used to measure reactive aldehydes in heart tissues homogenized in RIPA buffer. Bars are means ± SEM. * indicates significantly different from IR group ($p < 0.05$). (ANOVA, p= 0.0004; IR+Asc vs IR, p= 0.0006; IR+Cat vs IR, p= 0.0403; IR+Asc+Cat vs IR, p= 0.0006).

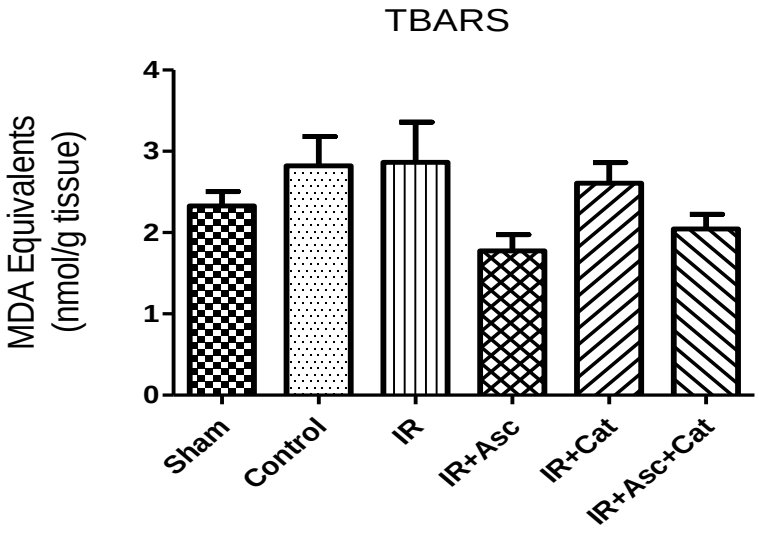


Figure 4.5 *Levels of TBARS in homogenates using 3% metaphosphoric acid (MPA).* The TBARS assay was used to measure reactive aldehydes in heart tissues homogenized in metaphosphoric acid buffer. Bars are means ± SEM.

4.3. Total MDA

To assess ischemia-reperfusion induced oxidative stress in cardiac cells, total MDA which is an indicator of oxidative stress was measured (Fig 4.6) using the colorimetric assay for total MDA (Gerrard-Monnier et al., 1998). Ascorbate showed more than 100% inhibition of lipid peroxidation, catechin showed 67% inhibition, while ascorbate plus catechin showed 71% inhibition compared to ischemia-reperfusion and sham groups.

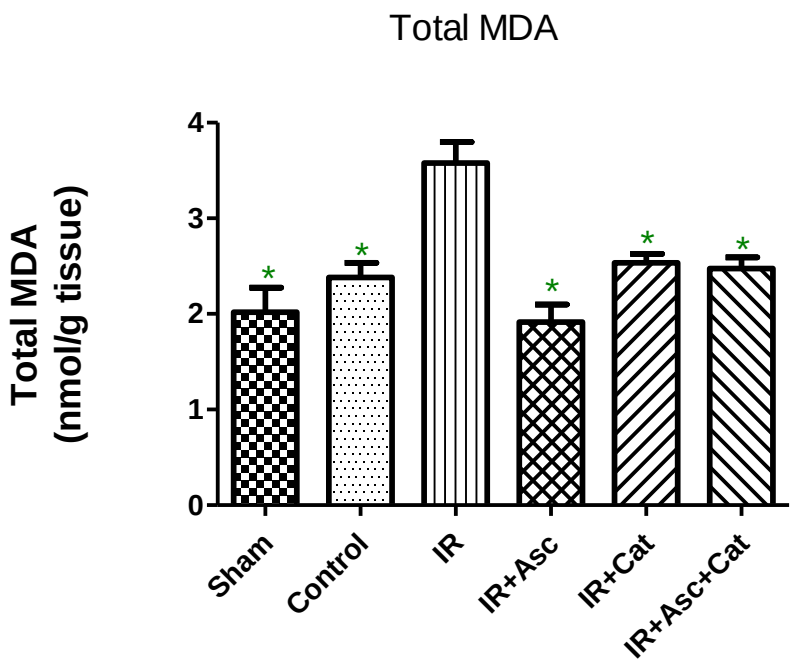


Figure 4.6 *Total malondialdehyde (MDA) in heart tissues*. The bars represent means ± SEM. * indicates significant difference from IR ($p < 0.05$). (ANOVA, p= 0.0001; Sham vs IR, p= 0.0001; Control vs IR, p= 0.0006; IR+Asc vs IR, p= 0.0001; IR+Cat vs IR, p= 0.0021; IR+Asc+Cat vs IR, p= 0.0011).

4.4. Caspase-3 activity

Apoptosis in the heart tissues was determined using examination of caspase-3 activity, one of the important effector caspases involved in the pathway of apoptosis. Ischemia-reperfusion increased the activity of caspase-3 activity (Fig 4.7). Ascorbate, catechin and ascorbate plus catechin gave up to 100% protection against caspase-3 activation compared to the IR and Control groups.

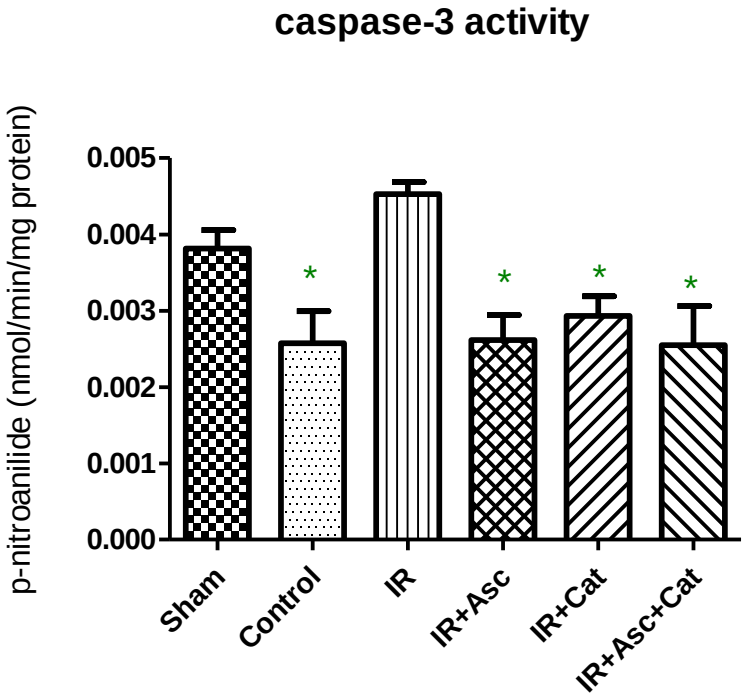
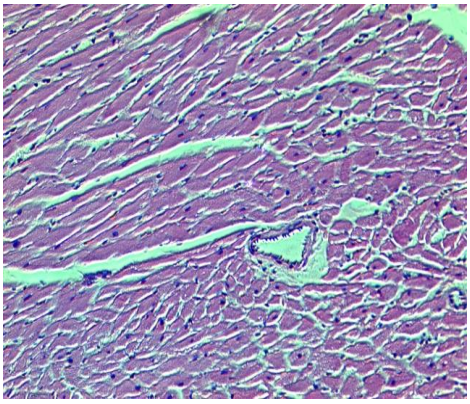


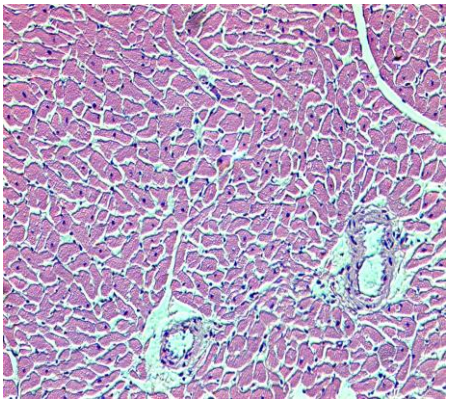
Figure 4.7 *Caspase-3 activity in heart tissues*. Caspase-3 activity was measured colorimetrically from cleavage of the substrate Ac-DEVD-p-nitroanilide. The bars represent means ± SEM. * indicates significant difference between IR, control and treatments groups ($p < 0.05$). (ANOVA, p= 0.0011; IR+Asc vs IR, p= 0.006; IR+Cat vs IR, p= 0.030; IR+Asc+Cat vs IR, p= 0.004; Control vs IR, p= 0.007).

4.5. Histological analysis using hematoxylin and eosin stain (H&E)

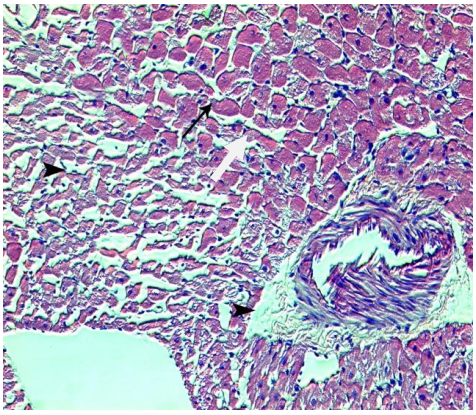
Light micrographs of H&E stained myocardium according to each group shows:



A) Sham heart



B) Control heart



C) IR heart

Figure 4.8 *Sham and Control groups: normal cardiac muscle fibers with centrally located nuclei (Fig 4.8 A and B) and IR group: myonecrosis (arrow) and edema (arrowhead) (Fig 4.8 C).*

In the Sham and Control groups, the heart showed normal myocardium with striated muscle fibers and centrally located nuclei (Figure 4.8 A and B). The IR group showed focal areas of degeneration and necrosis in the cardiac muscle fibers with loss of its striation (Figure 4.8 C). In this condition, the nuclei were pyknotic or completely absent, and the sarcoplasm became contracted and more eosinophilic (Figure 4.8 C).

For the H&E stain, only one rat per group was used, but the histopathological observations were graded by a blinded pathologist with regard to degeneration and necrosis in the cardiac muscles and edema. The pathologist grading of the histopathology in each of the hearts is shown in Table 1. Vitamin C treated heart showed mild degeneration, necrosis and edema, vitamin c plus catechin have also shown the same effect compared to the IR heart which showed severe degeneration, necrosis and edema.

Table 1. *Histopathological grading of hearts*. *(-) nil, (+) mild, (++) moderate, (+++) severe. Each heart was evaluated based on 3 images.

Groups	**Degeneration and necrosis**	**Edema**
Sham	-	-
Control	-	-
IR	++++	+++
Vitamin C	+	+
Catechin	++	++
Vitamin C & Catechin	+	+

CHAPTER 5: DISCUSSION

Although previous studies have looked at the protection against ischemia-reperfusion injury by ascorbate alone or flavonoids alone, most have used very high concentrations and no studies have looked at the possible cooperation of flavonoids and ascorbate in protecting from myocardial ischemia-reperfusion injury. We hypothesized that ascorbate can act synergistically with a relatively low level of catechin to protect the myocardium from ischemia-reperfusion injury.

5.1. Lipid Peroxidation

The results of the study herein showed that dissolving ascorbate powder directly in the perfusate following the induction of ischemia gave more than 100% significant protection against oxidative damage which occurs following ischemia-reperfusion, as assessed using thiobarbituric acid reactive substances (TBARS) and total malondialdehyde (MDA). Although TBARS is a well-established method to detect oxidative damage in tissues, it is not entirely specific to lipid peroxidation because other aldehydes and ketones can react. Ascorbate also gave 100% significant protection against oxidative stress when evaluated by total MDA.

Similar to our results, Molyneux et al. (2002) found that 15 min reperfusion of isolated rat hearts with 100 µM ascorbate after 45 min of ischemia reduced the levels of malondialdehyde using TBARS to levels similar to the control (a non-ischemic group perfused for the full 60 min time period). In addition, ascorbate supplementation to post reperfusion patients who experienced myocardial infarction showed complete restoration of malondialdehyde to normal levels (Bhakuni et al., 2006).

However our result with ascorbate is in disagreement with reports that administration of ascorbate, delivered during reperfusion had no effect on post-ischemic injury in isolated rat heart (Gao et al., 2002). The reason for this disagreement is not clear, but the reperfusion period used by Gao el al. (2002) might be the reason, which was 120 minutes, while in our study, it was only 90 minutes.

Catechin (5 µM) also decreased lipid peroxidation by 67% which was assessed by TBARS and total MDA. Similar to our results, Modun et al. (2003) has reported the ability of catechin to inhibit lipid peroxidation in heart ischemia-reperfusion with oral delivery of 250 mg pure catechin for 10 days prior to the induction of ischemia-reperfusion injury. In addition, Babu et al. (2006) have reported the protective effect of green tea extract in reducing lipid peroxidation in the hearts of streptozotocin induced diabetic rats following the delivery of 300 mg/kg per day for 4 weeks. These previous studies that tested the effect of catechin against lipid peroxidation have used high in vivo doses which allow accumulation in the tissues; our study shows directly the protection by catechin against lipid peroxidation at a level that might be achieved in blood in vivo, in an in vitro condition of myocardial ischemia-reperfusion.

Although ascorbate and catechin were effective in protecting the isolated rat heart against lipid peroxidation following ischemia-reperfusion injury, the complete protection provided by ascorbate alone did not allow observation of a synergistic effect when given together.

5.2. Cell apoptosis

In ischemia-reperfusion injury, cell death occurs by both necrosis and apoptosis (Zhao & Vinten-Johansen et al., 2002). In our study, caspase-3 activity was measured as an indicator of cell apoptosis.

Consistent with the results on lipid peroxidation, ascorbate inhibited the activation of caspase-3 significantly up to 100%, while catechin inhibited the activation of caspase-3 activity by 90%. Also similar to our lipid peroxidation results, ascorbate and catechin individually inhibited so strongly that no cooperation was observable when given together.

Several in vitro and ex-vivo studies have revealed the effect of catechins in limiting apoptosis and infarction size. Townsend et al. (2004) reported the effect of catechin after adding 100 μM to the perfusion buffer 30 minutes before the induction of ischemia and during perfusion for 60 minutes, where it reduced the percentage of infarction within the risk zone. Furthermore, Aneja et al. (2004) have reported that administration of 10 mg/kg/iv catechins following the induction of 30 minutes ischemia followed by continuous infusion of 10 mg/kg/h during two hours reperfusion period have shown protection against cell apoptosis.

Other studies have shown orally administered catechins to protect against heart reperfusion injury. In the study by Townsend et al. (2004) oral supplementation of catechin (100 μM in drinking water) for 7 days gave a significant decrease in caspase-3 activity in cardiac muscle following ex vivo ischemia-reperfusion. In another feeding study, Suzuki et al. (2007) reported the significant effect of catechin in reducing apoptosis and necrosis after feeding rats 20 mg/kg/day catechin for 14 days. Yanagi et al. (2011) showed that 1mmol/L drinking water of epigallocatechin-3-gallate (EGCG), which is the most abundant polyphenolic catechin in green

tea, reduced myocardial apoptosis by inhibiting caspase-3 activation in an isolated rat heart model. Akhlaghi and Bandy (2010) have also confirmed the protective effect of green tea extract supplied in the diet for 10 days which inhibited caspase-3 activation by 85% in hearts subjected to ex vivo ischemia-reperfusion.

To date, no study has tested the dose or the route of administration that was used in our study. In this study, only 5 µM of catechin was used and it was added to the perfusate following 30 min of global ischemia, while other in vitro studies have added catechin to the perfusate before induction of global ischemia. While adding catechin before and after ischemia might have stronger effect compared to adding it after the period of ischemia as we did in our study, our results show that the presence of a reasonably low amount of catechin in the perfusate only during reperfusion can give strong protection.

The protective effect of ascorbate in inhibiting caspase-3 activation has also been reported by other investigators; Ichiki et al. (2008) found that ascorbate treatment inhibited the activation of caspase-3 by reducing the apoptotic Kupffer cells in hepatic ischemia-reperfusion injury. Also, ascorbate was able to reduce caspase-3 activity during hypoxia-reperfusion in human endothelial cells (Dhar et al., 2005). To our knowledge, no study has tested the protective effect of ascorbate on apoptosis following myocardial ischemia-reperfusion injury. Our study is the first to report the effect of 150 µM ascorbate in reducing apoptosis (caspase-3 activity) following myocardial ischemia-reperfusion injury in an isolated rat heart.

5.3. Tissue glutathione levels

Despite the fact that the treatment groups had remarkably reduced oxidative damage and cell death, surprisingly, they did not inhibit a decline in cellular reduced glutathione (appeared to make it worse), nor improve the GSH to GSSG ratio, which is an important biomarker that reflects the cellular redox state. The level of glutathione disulfide (GSSG) in the hearts was also not significantly affected by ischemia-reperfusion or by any of the treatments, although there was a trend towards an increase with ischemia-reperfusion and a suggestion of protection by ascorbate and catechin.

The effect of ascorbate on glutathione levels in our study is in disagreement with reports from other investigators; Molyneux et al. (2002) has reported that ascorbate was able to keep the glutathione and glutathione disulfide levels similar to the control group values. The possible reason for this inconsistency is the reperfusion time used in Molyneux study which was 15 minutes reperfusion only, while in our study, the reperfusion time was 90 minutes. In hepatic ischemia-reperfusion injury, ascorbate was also able to preserve the glutathione levels similar to the control and sham operated groups (Korkmaz et al., 2009), but the dose of ascorbate in this study was 250 mg/kg (1.4 mM) injected intra-peritoneally 1 hour prior to ischemia, whereas in the current study, we only used 150 µM of ascorbate added directly to the perfusate for 90 minutes following 30 minutes of global ischemia.

Similar to ascorbate, catechin had no significant effects on GSH, GSSG, or GSH/GSSG ratio. Comparable to our results, Akhlaghi et al. (2010) has reported that feeding rat with green tea extract for 10 days prior to ex vivo ischemia-reperfusion had no significant effects on the GSH, GSSG, or GSH/GSSG ratio. Our results are in controversy with those of Yamazaki et al. (2008) which showed that oral gavage of 1mg/kg/day of epicatechin for 10 days prior to

coronary artery occlusion and 2 days during reperfusion in vivo significantly reduced the GSSG/GSH ratio compared to the ischemia-reperfusion control group which had a significant increase in GSSG/GSH ratio. An important difference when comparing our study with this study was that the epicatechin was delivered prior to and for 2 days continuously during the reperfusion period, which can influence the cellular redox state. In our study, catechin was delivered only for 90 minutes during the reperfusion period.

In addition, longer term in vivo delivery of catechins, unlike the current acute in vitro situation, may influence glutathione levels through effects on glutathione biosynthesis. In streptozotocin induced diabetic rats for example, delivery of 300 mg/kg per day of green tea extract for 4 weeks improved the antioxidant status by increasing glutathione levels significantly in the rat hearts (Babu et al., 2006).

The possible explanation for the lack of catechin effect on glutathione levels in our study may be due to the involvement of glutathione in detoxification reactions. Oxidized catechin has electrophilic properties, and can be detoxified through conjugation with glutathione which may affect the cellular glutathione level after being oxidized in oxidative conditions such as ischemia-reperfusion. Formation of catechin-glutathione adducts has been reported in the presence of peroxidase and hydrogen peroxide (Moridani et al., 2001). In addition, glutathione adducts and GSSG may be excreted from the myocardial cells into the perfusate effluent.

5.4. Histological analysis

The hematoxylin and eosin (H&E) stain was used in this study for histological analysis, only one rat heart per group was stained with H&E. The H&E stain is considered the routinely used stain in histopathology, which provides a more detailed view of the tissue by staining cell

structures including the nucleus, cytoplasm and organelles components. In this study, H&E stain has shown normal myocardium with striated muscle fibers and centrally located nuclei in the sham and control group, while the IR group has shown focal areas of necrosis and degeneration in the cardiac muscle fibers with loss of its striation. In an independent evaluation the ascorbate treated rat has shown less edema and degeneration compared to IR rat. Since there was only one rat per group, it is difficult to conclude that ascorbate was protective. It would be valuable to test ascorbate with the same dosage using H&E stain with larger sample of rats.

5.5. Evaluation of the IR Model

Although the Langendorff ischemia-reperfusion model is actively used as a useful method in cardiovascular research (Skrzypiec-Spring et al., 2007; Bell et al., 2011), controls for baseline (sham) and continuous perfusion are rarely used. Such controls establish validity of the model and show the real strength of any protection. For example, all of the previous research that were done on protection by catechins or ascorbate using this model of global ischemia were lacking the non-ischemia continuous perfusion group (Molyneux et al., 2002; Gao et al., 2002; Modun et al., 2003; Townsend et al., 2004 and Korkmaz et al., 2009), to indicate the influence of ischemia. Some had a baseline sham group, while others had no controls except for the IR group without treatment (Gao et al., 2002).

In this study, some of the measurements did not show a significant difference between the non-ischemia perfusion control group and the ischemia-reperfusion group. The glutathione levels in heart tissues, and also the TBARS levels showed a non-significant difference between these groups. The reason is not clear. However, a possible explanation would be due to the time of ischemia which was only 30 minutes and it is possible that 30 min was not enough duration to cause enough insult to the rat heart. For example, a study on global ischemia-reperfusion in

mouse hearts (Wang et al., 2001) showed increasing damage and loss of function with ischemic periods up to 60 min. But the fact that both groups have shown similar results for the glutathione and TBARS levels raise a concern regards the reliability of the IR model. Nevertheless, the measurements of MDA and caspases-3 activity showed significant differences between IR and perfusion controls in my study, and also showed significant protection by ascorbate and catechin.

5.6. Potential Limitations

One limitation was that the biochemical analyses in this study were performed on complete hearts and not selectively on the ischemic area. If the analyses could be performed more specifically on the area impacted in the IR group, larger differences could likely be observed.

Another limitation was that the experiments were conducted in healthy animals, while in the clinical situation, patients will likely be suffering from co-occurring diseases such as diabetes and hypertension.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

In conclusion, this study shows that ascorbate alone (or in combination with catechin); under the condition of ischemia-reperfusion injury gave a strong protection against cell death and oxidative damage. Based on the study results, ascorbate can be used clinically for patients undergoing angioplasty, cardiac surgery, or heart transplantation especially since ascorbate was delivered directly into the perfusate during the reperfusion period. While not tested in the current model, ascorbate might also be used in the diet for patients who experienced a heart attack.

This study also shows that at the dose of 5 μM, catechin significantly protected the heart against cell death and oxidative damage. Based on a review of the literature, this study is the first to report the protective effect of catechin using this dose. Ascorbate and catechin have shown promising results in protecting the heart against oxidative damage and cell death; however there was no evidence of any synergistic protective effect when ascorbate and catechin were given together.

6.2. Future directions

In future studies, one should consider comparing the protective effect of different classes of flavonoids and their potency against ischemia-reperfusion injury in an isolated rat heart using a dose-response curve to determine the best effective dose. This will show which class of flavonoid is the most potent.

Since, there was no synergistic protective effect when ascorbate and catechin were given together in the current study, it is highly recommended for future studies to lower the doses of ascorbate and catechin, because these doses had such strong effects alone. Also they are at the upper limit of dietary-relevant concentrations in blood. It is possible for example that 50 µM of ascorbate and 1 µM or less of catechin would show a cooperative effect when combined together.

Since the effect of 150 µM ascorbate has shown less edema and degeneration compared to IR rat based on the histological analysis using H&E stain in a sample size of n =1, it will be useful to test the same dose of ascorbate using triphenyl tetrazolium chloride (TTC) stain which is a well-known method to detect the infarction size in ischemic myocardium, to determine if ascorbate can reduce the infarction size significantly or not.

The time of global ischemia in this study was 30 minutes. Future studies should consider using 45 minutes of ischemia to cause more insult to the heart, which will help to evaluate the potency of using 150 µM of ascorbate following more stress on the heart. Furthermore, the time period between isolation of the heart and mounting the aorta should be limited to four minutes to avoid the damaging of the heart if the hypoxic duration is too long, and the potential effect of ischemic preconditioning if perfusion is delayed.

The effect of ascorbate and catechin should be tested in an in vivo experiment as well to compare it with the ex-vivo results. It will also be useful if future studies can test the same dose of ascorbate and catechin in clinical situations such as angioplasty and heart transplantation if added directly into the perfusate during the reperfusion period.

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