University of Alberta

Docosahexaenoic Acid Induced Apoptosis In H9c2 Cells And Changed Cardiac Function After Ischemia-Reperfusion Injury

by

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Dedication

I dedicate this thesis to my lovely parents, Dr. Suhail Qadi and Azza Al-Rifaie, and my husband, Rani Al-Sairafi, for their support and encouragement, and for advising me to work hard; and my lovely daughters, Raghad and Retal, who kept me busy from working on my thesis. I also dedicate this thesis to my sisters, Roha and Noha, and my brother, Gaith, who always give me the strength to keep working to make my dreams come true. I also dedicate this work to my supervisor, Dr. John Seubert, for supporting me and reminding me to work hard, by always saying "Nice work. It's coming."

Abstract

Cardiovascular disease (CVD) remains one of the leading causes of death worldwide. As such, a vast amount of research has investigated novel therapies for preventing and/or reducing CVD. Much evidence has demonstrated the importance of dietary composition in increasing or lowering risks of CVD. While the role played by dietary n-3 polyunsaturated fatty acids (PUFAs) in reducing CVD has been recognized for many years, the protective mechanisms of these molecules, notably toward ischemia-reperfusion (IR) injury, remain unknown. Ecosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3, DHA) are two of the most important n-3 PUFAs. Both are present in fish oil. The objective of this study is to investigate the effect of acute administration of DHA on IR injury. **Methods:** Hearts from male C57BL6 mice were isolated and perfused in Langendorff mode and then subjected to IR injury. Hearts were perfused with different concentrations of DHA (0, 10, 50 and 100 μM) to determine its effect on cardiac function and recovery. Mechanistic studies were performed using rat myoblast cells (H9c2 cells) in an anoxia-reoxygenation protocol. Cell viability (MTT assay), cytochrome c release, and caspase-3 and caspase-8 activities were measured to compare cellular injury in DHA treated cells versus controls. The impact of DHA on mitochondrial morphology and function was assessed using epifluorescent microscopy. **Results:** Data demonstrated that DHA has adverse effects on both pre- and post-ischemic left ventricular developed pressures and on the heart rate, systolic and diastolic heart rates. Cell experiments revealed that significant cell death occurs in a concentration-dependent manner when H9c2

cells are treated with DHA and subjected to anoxia-reoxygenation injury. Moreover, apoptotic cell death is caused by DHA treatment, during which cytochrome c is released and caspases-8 and -3 are activated. Significant mitochondrial fragmentation and loss of membrane potential were observed with high concentrations of DHA. **Conclusion:** Our data suggest that acute treatment with DHA impedes the function of isolated hearts and triggers apoptotic cell death.

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List of Abbreviations

20-HEPE	20-Hydroxyeicosapentaenoic Acid
20-HETE	20-Hydroxyeicosapentaenoic Acid
22-HDoHE	22-Hydroxydosahexaenoic Acid
AA	Arachidonic Acid
AF	Artrial Fibrillation
AHA	American Heart Association
ALA	Alpha Linolenic Acid
ATCC	American Type Culture Collection
ATP	Adinosine Triphosphate
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
COX	Cyclooxygenase
CVD	Cardiovascular Disease
CYP	Cytochrome P450
DD	Death Domain
DED	Death Effector Domain
DHA	Docisahexaenoic Acid
DMEM	Duldecco's Modofied Eagle Medium
DMSO	Dimethyl Sulfoxide
Dox	Doxorubicin

DRP1	Dynamic-Related Protein-1
EDP	Epoxydocosapentaenoic Acids
EEQ	Epoxyeicodtateraenoic Acid
EET	Epoxy-Eicoatrienoic Acids
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulaum
ETC	Electron Transport Chain
FADD	Fas-Associated Via Death Domains
FasL	Fas Ligand
FBS	Fetal Bovine Serum
Fis1	Fission 1
HHE	4-Hydroxy-2-Hexenal
HNE	4-Hydroxy-2-Nonenal
HUVEC	Human Umbilical Vein Endothelial Cells
ICM	Ischemic Cardiomyopathy
IHD	Ischemia Heart Disease
IM	Inner Membrane
IMS	Intermembrane Space
IPC	Ischemic Preconditioning
IR	Ischemia Reperfusion
LA	Linoleic Acid
LAD	Left Anterior Desending
LOX	Lipoxygenases

LTs	Leukotrienes
LVDP	Left Ventricular Developed Pressure
LVP	Left Ventricular Pressured
MFN	Mitofusins
MI	Myocardial Infarction
mNCX	Mitochndroal Sodium Calcium Exchanger
mPTP	Mitochondrial Permeability Transition Pore
MTT	3-(4,5 Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide
OM	Outer Membrane
Opa 1	Optic Atrophy 1
PBS	Phosphoate Buffered Saline
PCD	Programmed Cell Death
PE	Phosphatidylthanolamine
PFA	Paraformaldehyde
PGs	Prostaglandins
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PS	Phospholipidserine
PUFA	Ployunsaturated Atty Acid
SR	Sarcoplasmic Reticulum
TBARS	Thiobarbituric Acid Reactive Substances

TMRE	Tetramethylrhodamine Ethyl Ester
TNF-alpha	Tumar Necrosis Factor Alpha
TNFR-1	Tumer Necrosis Factoe Alpha Receptor-1
TXBs	Thromboxane
VLDL	Very-Low-Density Lipoprotein

Chapter 1

Introduction

1.1 Cardiovascular Diseases and Cardioprotection

Cardiovascular diseases (CVDs), such as atherosclerosis, high blood pressure, coronary heart disease and stroke, are the leading cause of morbidity and mortality in many developed countries, including Canada (Menesi et al., 2009; Petrovski et al., 2011; Frank et al., 2012; Gerczuk and Kloner, 2012). According to the Heart Stroke and Foundation of Canada (2008), over 69,500 deaths (29%) have occurred as a result of CVDs. Of these deaths, 54% are attributed to ischemic heart disease (IHD). Many factors, such as dyslipidemia, hypertension and hyperglycemia, are major risks for the development of altered structures and functions that cause thickness of the arterial wall, leading to IHD. Because of the high incidence of CVD in both western and developing countries, the need for research remains important in global efforts to reduce heart disease (Chiong et al., 2011; Harvey and Leinwand, 2011).

Coronary artery disease (CAD) is a CVD that requires attention because it causes the hospitalization of many patients worldwide. Ischemia occurs as a result of insufficient blood supply to the heart. The root cause of the disease is plaque that occurs in the coronary artery, leading to atherosclerosis, which reduces the blood supply to the heart. Reperfusion is the term used to describe the sudden flow of blood to the heart after ischemia. In the first few seconds of reperfusion, mitochondria begin to produce energy at a level similar to that pre-ischemia. Both ischemia and reperfusion can damage the heart or cause dysfunction to its tissues, a condition called ischemia-reperfusion (IR) injury (Powers et al., 2007; Raedschelders et al., 2012). The damage that occurs to the heart depends on the

length of ischemia and reperfusion. For example, when ischemia lasts for 5 to 20 minutes, the injury could be reversible with no occurrence of cell death. However, if the ischemic period lasts longer than 20 minutes, damage to cardiac myocytes is irreversible, leading to cell death (Powers et al., 2007). At the initial onset of an ischemic event, usually lasting up to 20 seconds, the only available source of energy is anaerobic glycolysis. After 60 to 90 minutes of coronary occlusion, with no production of ATP by glycolysis, heart contractions slow down significantly. Further studies on the important role of anaerobic glycolysis in ischemia must be conducted (Frank et al., 2012).

The heart is a complex network of cells that must function properly to prevent many health disorders. Cardiomyopathy may occur when the heart fails to tolerate different pathological events and can generally be caused either by a genetic mutation, which results in hypertrophic cardiomyopathy, or stress and certain chemotherapeutic agents, which result in acquired cardiomyopathy (Harvey and Leinwand, 2011). Although many pharmacological therapies have been developed to limit the occurrence of CADs, such as ischemic cardiomyopathy (ICM), they remain the leading cause of death in most developed countries (Templin et al., 2011). Surgical treatment for ischemic heart failure shows definite ICM as a massive reduction in left ventricular function resulting from CVD (Schuster et al., 2012). The heart has some ability to regenerate and repair itself in case of stress. Proper maintenance of the functions of the heart is necessary to prevent the development of heart disease (Chiong et al., 2011).

The term “cardioprotection” refers to therapeutic strategies that limit or prevent heart damage. These therapeutic interventions include primary or secondary techniques targeting acute myocardial infarction (Kubler and Haass, 1996). A proper lifestyle, regular exercise and diet are important strategies for maintaining a healthy heart (Massaro et al., 2008; Lavie et al., 2009; Petrovski et al., 2011). Conditioning is another powerful cardioprotective strategy that strengthens the heart, making it more injury-resistant, as shown by Murry et al. (1986). Brief non-detrimental episodes of ischemia or pharmacological mimetics given prior, post or remote to a prolonged ischemic event can initiate signalling pathways that protect the myocardium. Preventing cell death that occurs as a result of reperfusion injury is considered the major impact of ischemic preconditioning. Many factors, such as autacoids (e.g., bradykinin, adenosine, opioids), kinase signaling pathways and mitochondria modulation (Murry et al., 1986; Yang et al., 2010; Minamino, 2012), play a role in ischemic preconditioning. Researchers believe that the benefits of ischemic post-conditioning lie in mechanisms that prevent changes in the intracellular pH and limit the production of reactive oxygen species (ROS). A possible mechanism of cardioprotection in ischemic conditions from distal organs to the heart involves the release of autacoids from the organ that activate kinase signalling pathways (Minamino, 2012). Unfortunately, both early reperfusion therapy and cardioprotective drugs given prior to ischemia are limited in clinical application as patients typically present after the onset of ischemia and/or are unable to reach medical facilities. As such, extensive work needs to be done to develop

therapeutic interventions to reduce or prevent damage caused by ischemia and reperfusion (Yang et al., 2010).

1.1.1 Myocardial ischemia and reperfusion injury

The heart needs energy to remain beating and functioning properly. Mitochondria are organelles responsible for the production of ATP, which provides the heart with energy and allows it to contract and relax (systole and diastole, respectively). Mitochondria represent over one third of the structure of the heart to provide the required energy. Changes that occur in the mitochondrial structure or function could be a sign of a developing CVD, such as heart failure or CAD (Ong and Hausenloy, 2010; Carreira et al., 2011; Kadenbach et al., 2011; Dedkova and Blatter, 2012).

Cardiomyocytes are involved in a cycle of excitation and contraction. During a cardiac cycle, Ca^{2+} is released via Ca^{2+} channels from the sarcoplasmic reticulum (SR) to increase cytosolic Ca^{2+} concentrations [$(Ca^{2+})_i$]. Mitochondrial Ca^{2+} uptake activates Ca^{2+}-dependent pyruvate dehydrogenase (Pepe, 2005) and efflux by the sodium calcium exchanger (mNCX). The mitochondrial membrane potential ($\Delta\Psi_m$), which is essential in energy regulation, plays a key role of oxidative phosphorylation. Pyruvate and fatty acids undergo biological reactions in the mitochondria to achieve reduced states of $NADH/NAD^+$ and $FADH_2/FAD$ via the electrochemical proton gradient pump with membrane-bound ATP synthase, during with greater increases in ATP synthesis occur (Kadenbach et al., 2011; Griffiths, 2012).

Blocking the supply of oxygen to the heart during ischemia will negatively

impact the mitochondria and causes reduction in ATP production. In this case, the compensatory source of energy is anaerobic glycolysis (Menesi et al., 2009; Kadenbach et al., 2011). Glycolysis causes lactate formation, cytosol acidification and sarcolemmal Na^+/H^+ exchanger activation. The ratio of ATP to ADP decreases as a result of Na^+/K^+-ATPase inhibition, leading to increased intracellular Na^+ followed by an increase in $[Ca^{2+}]_i$, which is affected by the mNCX (Cadenas et al., 2010; Kadenbach et al., 2011). When the ischemic period is prolonged, an increase in the intracellular Ca^{2+} and depletion of the compensatory source of ATP production will lead to a gradual loss of cellular integrity (Cadenas et al., 2010).

Restoring the blood supply after ischemia by reperfusion could reduce tissue damage. However, during reperfusion, undesirable changes to the cell functions, specifically those of mitochondria, could occur. One of the main causes of damage to the heart after reperfusion is mitochondrial generation of ROS that cause potential dysfunction to the cell. Besides ROS production, increased concentrations of intracellular calcium as a result of a leakage during mitochondrial permeability transition pore (mPTP) opening and loss of $\Delta\Psi_m$ could also occur (Cadenas et al., 2010; Nishida et al., 2010; Kadenbach et al., 2011).

1.2 Role of Mitochondria

Mitochondria play a significant role in intracellular energy metabolism, as well as essential roles in regulating cell survival and death. The word “mitochondria” comes from the Greek words “mito,” which means thread, and

"chondron," which means grain, the latter referring to the shape of the organism as observed under a light microscope (Nishida et al., 2010; Ong and Hausenloy, 2010; Ryu et al., 2010). Significant improvements in mitochondrial imaging have been achieved since the development of confocal microscopy and specific fluorescent probes, aiding in the understanding of mitochondrial morphology, function and dynamics. The mitochondrial structure consists of two separate functioning membranes, an outer membrane (OM) and an inner membrane (IM), an inter-membrane space (IMS) and the matrix compartment. Significant changes and/or damage to the mitochondrial structure result in cellular dysfunction that ultimately can result in disorders such as cardiomyopathies, cancer and obesity (Ong and Hausenloy, 2010; Nunnari and Suomalainen, 2012).

Mitochondria are known as "the energy power-plants" of cells because of their ability to produce the majority of the ATP required for cell functions. The outer and inner membranes of the mitochondria have ion channels that are essential in cellular function. Scientists have recently targeted mitochondria for novel therapies in cardiovascular and cancer research. However, many questions about the physiological role of the ion channels of mitochondria remain unanswered (Nishida et al., 2010; Ryu et al., 2010).

The mPTP is a non- specific channel that opens when the mitochondrial inner membrane becomes more permeable. Increases in mitochondrial permeability allow molecules of 1.5 kDa to exhibit, and rapid swelling occurs as a result of Ca^{2+} induction. The pore consists of an outer membrane with a voltage-dependent anion channel (VDAC) in the outer membrane, while the inner

membrane has adenine nucleotide translocase (ANT) and cyclophilin D (Cyp D). Maintenance of the opening of the mPTP depolarizes the mitochondria and limits ATP production by preventing mitochondrial repolarization (Halestrap, 2009; Nishida et al., 2010; Ryu et al., 2010). Metabolites and ions are essential for maintaining the ΔΨm and pH gradient for oxidation phosphorylation and ATP synthesis. The mitochondrial IM is impermeable to most metabolites and ions. One complex in the mitochondrial IM respiratory chain is the electron transport chain (ETC). The ETC moves H^+ from the mitochondrial matrix to the IMS using free energy released during electron transport. ΔΨm also plays a role in Ca^{2+} uptake to the mitochondria (Perry et al., 2011; Dedkova and Blatter, 2012).

Mitochondria are highly dynamic organelles that undergo a continuous balance of cycles of fusion and fission to maintain their integrity. Dynamic-related protein-1 (DRP-1) and fission 1 (Fis1) are the proteins that regulate mitochondrial fission, while mitochondrial fusion is regulated by mitofusins -1,-, mitofusin-2 (MFN-1,-2) and optic atrophy 1 (Opa 1) (Chiong et al., 2011; Nunnari and Suomalainen, 2012). Maintenance of the proper function and structure of the mitochondria is related to healthy cells and/or organs. In normal cells, it is essential to maintain the events of fission and fusion balance. Loss of balance between these events could lead to mitochondrial fragmentation and, down-regulation of Opa 1, activation of caspases and induction of cell death (Chen and Knowlton, 2011). Any mitochondrial dysfunction could be associated with several disorders, such as cardiomyopathies and cancer. Studies have yielded some evidence that indicates that essential mitochondrial functions could rely on

the level of activity of mitochondrial dynamics (Liesa et al., 2009; Ryu et al., 2010).

1.3 Polyunsaturated Fatty Acids (PUFAs)

Fatty acids are alpha carboxylic acids containing 4 to 24 carbon atoms and a long aliphatic tail. They are either saturated or unsaturated. Fatty acids are vitally important to cells, tissues and organs as they can be used as fuel and regulators of protein synthesis. They are key components of membranes, dictating characteristics such as fluidity, permeability and dynamics (Covington, 2004; Adkins and Kelley, 2010). As well, fatty acids can act as potent lipid mediators, regulating intracellular metabolic pathways and inflammatory responses. Conventional fatty acid nomenclature is based on the number of carbon atoms, the number of double bonds and the position of the first double bound. For example, oleic acid, a monounsaturated fatty acid, has 18 carbon atoms and one double bound found at carbon 9 (18:1n-9) (Harris et al., 2008; Gleissman et al., 2010; Serini et al., 2011).

Polyunsaturated fatty acids (PUFAs) contain multiple double bounds and are considered essential fatty acids because humans require them for cellular structure and function. PUFAs play an essential role in the growth and development of many organs, including the brain and heart (Leaf, 2008). However, mammals lack the ability to synthesize most of PUFAs endogenously because they do not have the enzymes necessary to form double bonds (desaturation) or add carbon atoms (elongation) to the fatty acid chains. Therefore, the only source of most PUFAs is nutrition (Adkins and Kelley, 2010; Westphal et al., 2011). N-3 PUFAs, including

α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are a major group of PUFAs that have a double bond at carbon position 3 (omega). Another important group of PUFAs is n-6-PUFAs, which have a double bond at carbon 6. Examples of these PUFAs include linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6) (Harris et al., 2008; Serini et al., 2011). In most Western societies, the ratio of n-6 PUFAs to n-3 PUFAs is high (approximately 10-20:1), correlating well with adverse health indications, such as coronary heart disease, inflammatory disease and cancer (Siddiqui et al., 2008a; Adkins and Kelley, 2010). Studies have suggested that changing one's diet and lifestyle to reduce the ratio of n-6 PUFAs:n-3 PUFAs will benefit overall health. For example, in countries such as Japan, where a significant amount of fish is consumed, the risks of cardiac disease and cancer are lower than those in countries that consume food with high ratios of n-6 PUFAs:n-3 PUFAs (Lavie et al., 2009; Lecerf, 2009; Gleissman et al., 2010).

1.3.1 Metabolism of N-3 PUFA

α-Linoic acid (ALA) and linoleic acid (LA) are the parent PUFAs of n-3 and n-6 PUFAs, respectively (Adkins and Kelley, 2010). ALA undergoes Δ6 desaturation, elongation and Δ5 desaturation to form EPA, which is further metabolized by Δ6 desaturation and elongation to yield DHA. Similar enzymes used to metabolize n-3 PUFAs are also used to metabolize n-6 PUFAs. LA is metabolized by Δ6 desaturation, elongation and Δ5 desaturation to produce arachidonic acid (AA) (Leaf, 2008; Siddiqui et al., 2008b; Adkins and Kelley, 2010). Most of the PUFAs are widely present in normal diets; nuts, seeds and

vegetable oil are main sources of LA. ALA is found in seeds of flax, rape and soybean oil, as well as in leafy green vegetables, while the best source of EPA and DHA is deep ocean fish, such as tuna, salmon and sardines. Cod liver oil is also an excellent source of EPA and DHA (Lavie et al., 2009)

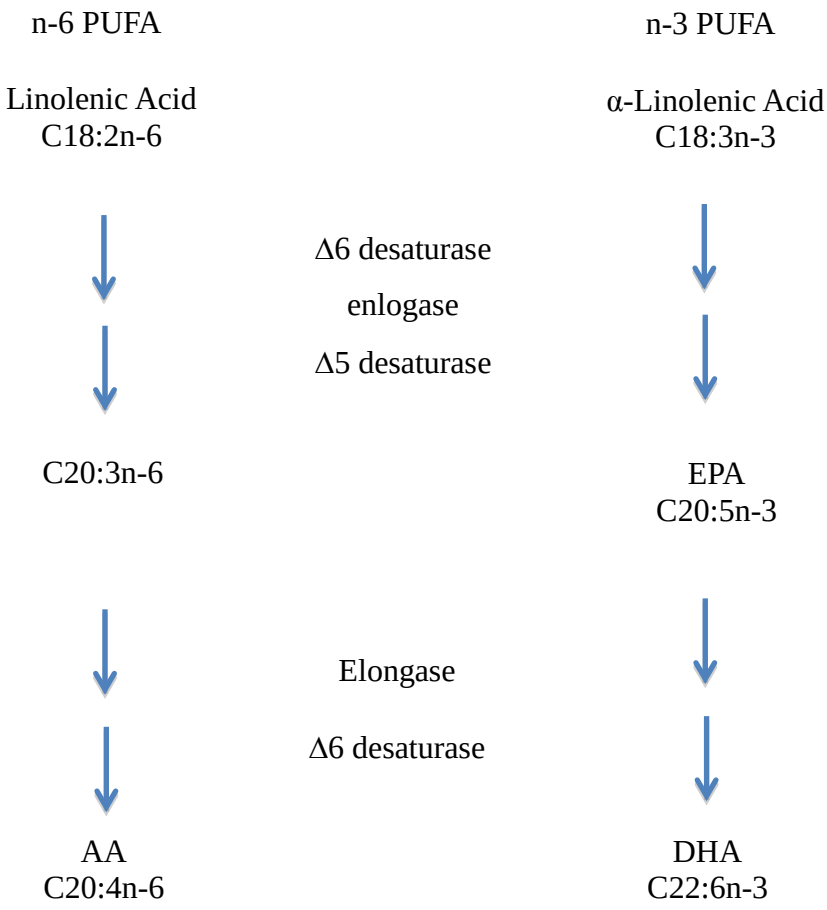


Fig.1.1 Synthesis of n-3 and n-6 PUFAs (adapted from (Siddiqui et al., 2008b))

Three enzymes are involved in the oxidation of PUFAs in the phospholipid membrane: cyclooxygenase (COX), lipooxygenases (LOX) and cytochrome P450 monooxygenases (CYP) (Konkel and Schunck, 2011). COX products are involved in inflammatory, thromboregulatory and chemotaxic responses. LOX modulates vasoconstriction and vascular permeability and CYP generates biologically active metabolites known as eicosanoids (Halestrap, 2009; Greene et al., 2011). AA is considered the most important physiological n-6 PUFA and is metabolized by COX to form prostaglandins (PGs) and thromboxane (TXB_2). In normal physiological conditions, PGs and TXB_2 play roles in vascular tone and hemostasis. In pathological conditions, COX-2 products are pro-atherogenic and pro-inflammatory, increasing risks of developing CVD. On the other hand, LOX enhances the formation of leukotrienes (LTs) from AA, which have been recently found to be involved in atherosclerosis (Siddiqui et al., 2008a; Siddiqui et al., 2008b; Janakiram et al., 2011). EPA and DHA compete with AA for binding and metabolism by COX and LOX to form resolvins and protectins. Resolvins play a role in inflammation resolution (Greene et al., 2011). Numerous studies have shown that n-3 PUFAs are able to inhibit the biosynthesis products of AA by altering the products and actions of its metabolites. For example, EPA is capable of lowering pro-aggregatory properties (Arnold et al., 2010a; Greene et al., 2011; Westphal et al., 2011).

CYP is an enzyme, also known as the “third branch of eicosanoids formation”, responsible for the formation of different bioactive metabolites. CYP-catalyzed hydroxylation and epoxidation of PUFAs depend on the chain length

and double bond structure (Halestrap, 2009). CYP2C and CYP2J are examples of specific isozymes responsible for epoxygenating AA to epoxyeicosatrienoic acids (EETs), as well metabolizing EPA to epoxyeicodtateraenoic acids (EEQs) and DHA to epoxydocosapentaenoic acids (EDPs). AA, EPA and DHA are hydroxylated by CYP4A and CYP4F to form 20-hydroxyeicosatetraenoic acid (20-HETE), 20-hydroxyeicosapentaenoic acid (20-HEPE) and 22-hydroxydocosahexaenoic acid (22-HDoHE), respectively. Over the last three decades, many researchers have studied the impact of parent PUFAs and their derived metabolites as therapeutic targets in the treatment or prevention of many CVDs (Halestrap, 2009; Greene et al., 2011; Westphal et al., 2011)

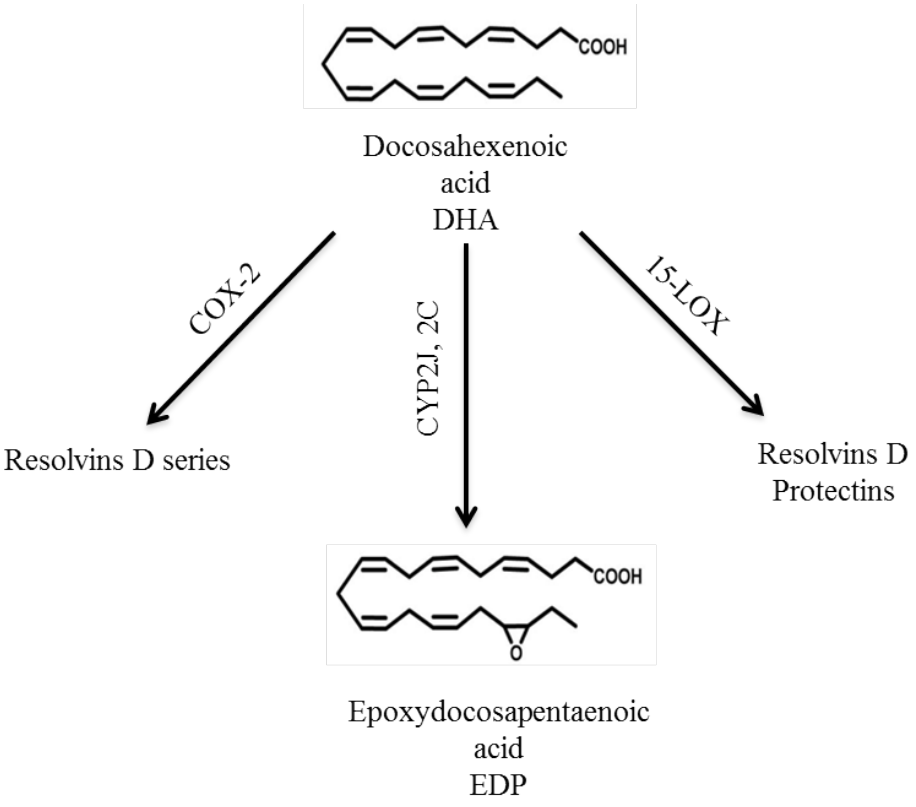


Fig.1.2 Metabolic pathway of n-3 by enzymatic oxidation (adapted from (Siddiqui et al., 2008b)).

1.3.2 Docosahexaenoic acid (DHA)

DHA, which is found in many biological systems, is considered the longest PUFA. It contains 22 carbon atoms and 6 double bonds. The main source of DHA is food containing high concentrations of n-3 PUFAs, such as fish. DHA is also available in dietary supplements. DHA is enriched and embedded in the cell membrane, specifically in phospholipidserine (PS) and phosphatidylthanolamine (PE) in the position sn-2 (Stillwell and Wassall, 2003). Because of the many double bonds in its structure, DHA significantly causes changes to the intrinsic properties of the membrane and activities of the membrane proteins (Adkins and Kelley, 2010). As a result, many biological functions are regulated by DHA, including lipid metabolism, homeostasis and cell differentiation and death (Gleissman et al., 2010). A recent study showed dose-dependent increases in plasma DHA as a result of ingestion of 2 g/day DHA for 1 month. The researchers of this study also found that DHA plasma concentrations remain high even after 6 months after dosing is ceased (Serini et al., 2011).

While many studies have provided evidence of the role of PUFAs in limiting the risk of different CVDs, other studies have looked at PUFA metabolites in the context of cardiac disease prevention. These studies investigated the roles of EEQs and EDPs in neonatal rat cardiomyocytes. Results show that these metabolites have an antiarrhythmic agent function (Arnold et al., 2010b). Another

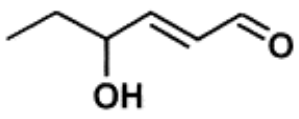
study shows that EDP is able to dilate coronary arterioles and small arteries of coronary smooth muscle cells to prevent hypertension (Ye et al., 2002). Evidence has also shown that EDPs contribute to platelet aggregation in the plasma of healthy humans (VanRollins, 1995). Therefore, EDPs are just as important in CVD prevention as its parent compound (DHA).

1.3.3 N-3 PUFAs and peroxidation

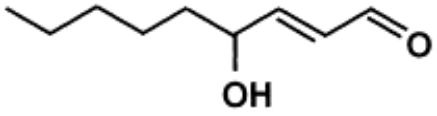
Dietary consumption of n-3 PUFAs for health benefits has drawn the attention of many scientists over last decade. Many concerns have been raised regarding the potential of adverse effects of n-3 PUFAs on human health (Riediger et al., 2009; Serini et al., 2011). For example, studies have shown a correlation between pro-hemorrhagic events and high amounts of n-3 PUFAs. High amounts of n-3 PUFAS have also been shown to cause oxidation (Russo, 2009). Oxidation takes place is depending on how the fish is stored before ingestion. Oxidation can occur either before or after fish consumption. However, debates about whether n-3 PUFAs have pro-oxidant or anti-oxidant properties continue (Serini et al., 2011).

Inequity between prooxidant/antioxidant systems will lead to cellular oxidative stress that causes changes in lipids and proteins (Catala, 2009). Lipid peroxidation causes the formation of free radicals that will subsequently damage cellular components. Oxidative damage that occurs in the mitochondria and leads to the formation of ROS could be one of the main causes of human disease, such as CHD, inflammatory disorder and neurodegenerative diseases (Yang et al., 2003; Uchida, 2007). PUFAs present in membrane phospholipids are more

susceptible to peroxidation, which can alter the fluidity and the permeability the membrane. The main lipid peroxidation product of n-6 PUFAs and n-3 PUFAs are known as 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE), respectively. Evidence suggests that oxidative stress enhances the release of HNE, which has a pathophysiological effect on cells and tissues (Uchida, 2003; Catala, 2009). As the number of double bonds increases in the structure of the fatty acids, so will the susceptibility to peroxidation. Therefore, DHA having the longest carbon chain and 6 double bonds is more susceptible to peroxidation, which can result in a major alteration in the structure of the fatty acid, such as a double bond loss or rearrangement (Awasthi et al., 2003; Uchida, 2003; Long et al., 2008; Catala, 2009).



HHE



HNE

Food that contains antioxidants, as well as high amounts of n-3 PUFAs, can better prevent health problems than food with high amounts of n-3 PUFAs and no antioxidants. Consuming food containing high amounts of oxidative products from n-3 PUFAs but without antioxidants poses potential problems (Serini et al., 2011). Evidence suggests these problems may be alleviated by co-administration of n-3 PUFAs with high amounts of antioxidants, such as α-tocopherol. The recommended dose for preventing primary and secondary CVD is

0.5 g/day to 1.0 g/day (Lavie et al., 2009). Any increase in the suggested amount of n-3 PUFAs could increase the likelihood of oxidation. An earlier study has shown that DHA supplementation of 0.8 g/day to 1.6 g/day for 2 weeks in healthy adult males increases HHE levels in plasma and red blood cells. However, sufficient work to determine the n-3 PUFA concentration that may be considered toxic has not been done (Serini et al., 2011).

1.4 Role of N-3 PUFA in Cardioprotection

Beginning in the 1970s, numerous epidemiological studies have investigated the association between CVD and the consumption of fish oil. Results have shown reductions in the risk of many CVDs (Lavie et al., 2009; Le Guennec et al., 2010). The most notable data were obtained from studies investigating populations that had a major dietary component of n-PUFAs (fish), such as the Japanese and Greenland Inuit. In a typical Western diet, significantly higher amounts of n-6 PUFAs are consumed compared with n-3 PUFAs (Russo, 2009). The ratio of n-6 PUFAs to n-3 PUFAs in Western diets is 10:1 instead of the recommended ratio of 1:1 (Lee et al., 2008). The American Heart Association (AHA) recommends eating fish approximately twice a week or 1 g per day; this amount of fish is predicted to lower the CVD mortality rate by 29% (Covington, 2004; Shukla et al., 2010). To date, the mechanisms behind beneficial effects of n-3 PUFAs remain unknown (Covington, 2004; McLennan et al., 2007; Lavie et al., 2009; Le Guennec et al., 2010).

Increasing the phospholipid contents of n-3 PUFAs, especially those of EPA and DHA, is believed to alter the membrane structure and biochemistry to

beneficially impact cardiovascular physiology, thus lowering CVD morbidity (Carroll and Roth, 2002). N-3 PUFAs can reduce cardiac arrhythmias and lower atherogenesis because they limit the risk factors of atherothrombosis by reducing platelet aggregation, lowering blood pressure and reducing plasma triglycerides (Covington, 2004; Lee et al., 2008). Numerous studies have proven the important role of n-3 PUFAs in reducing arrhythmias. These studies suggest that n-3 PUFAs inhibit voltage-gated sodium, L-type calcium and delayed rectifier potassium channels to prevent arrhythmia (Lavie et al., 2009). Another proposed role of n-3 PUFAs is prevention of cytosolic calcium overload following ischemic injury (Massaro et al., 2008). Several studies have examined the effect of EPA and DHA in hyperlipidemia and indicated that n-3 PUFAs are associated with reduced plasma triglyceride levels. N-3 PUFAs have the ability to suppress the synthesis of very-low-density lipoprotein (VLDL) and cholesterol (Covington, 2004). N-3 PUFAs reduce proinflammatory cytokines, such as tissue necrosis factor α, interleukin 6 and interleukin 1β (Lee et al., 2008). Taken together, the evidence suggests the beneficial effects of n-3 PUFAs for the primary or secondary prevention of variable heart diseases.

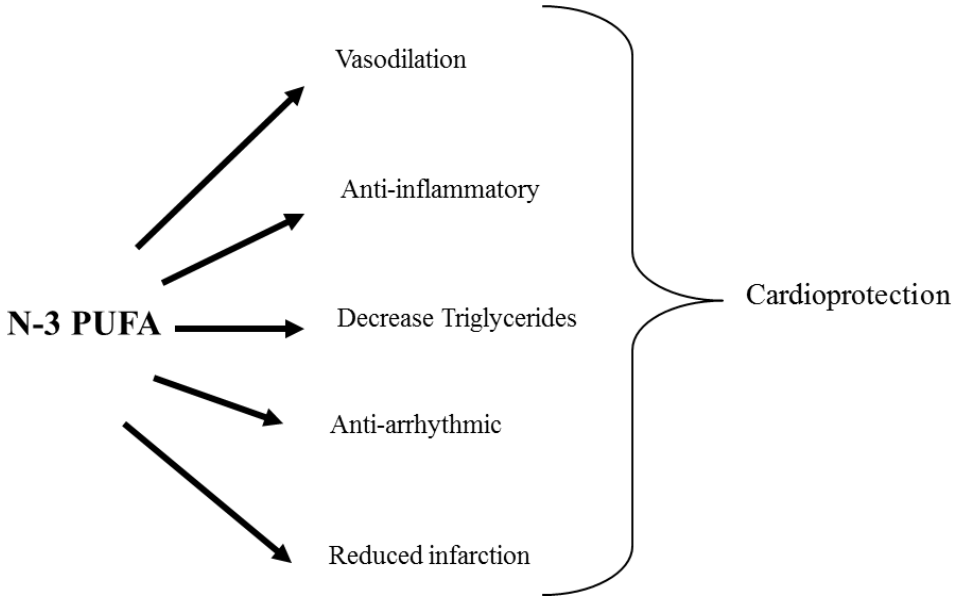


Fig.1.3 Role of n-3 PUFAs in limiting cardiovascular diseases (adapted from (McLennan et al., 2007)).

1.5 Effects of N-3 PUFA

In addition to lowering the risk of CVDs, literature has shown a correlation between increased consumption of n-3 PUFAs (specifically fish oil) and a lower incidence of cancer (Kremmyda et al., 2011). However, the molecular mechanism(s) for this benefit remains unknown. It has been reported that n-3 PUFAs can alter the structure and function of mitochondria. Some of these alterations are due to cytotoxicities of lipid peroxides derived from DHA, which can lead to cell death. Tests conducted on a human colon tumour cell line and rat carcinosarcoma have also shown that when n-3 PUFAs increase the mitochondrial Ca^{2+} level, a corresponding increase in the ROS release may be observed (Siddiqui et al., 2008a; Gleissman et al., 2010). Evidence has indicated that n-6 PUFAs are associated with induction of tumour formation. However, n-3 PUFAs are associated with preventing tumour formation (Spencer et al., 2009; Siddiqui et al., 2011). The products of eicosanoids derived from n-3 PUFAs could play a role in inhibiting the growth of colon cancer and breast cancer. Data have also shown that fish oil is able to lower the risk of pancreatic cancer development (Kremmyda et al., 2011).

Some studies have shown that the low intake of n-6 PUFAs and high intake of n-3 PUFAs will significantly reduce morning stiffness and relieve tender, swollen joints by lowering the inflammatory mediators of rheumatoid arthritis, which is extremely beneficial for patients suffering rheumatoid arthritis (Covington, 2004). Other epidemiological evidence indicates the preventive role of n-3 PUFA consumption in risks of Type 2 diabetes. Studies on children at high

risk for Type 1 diabetes recommend administration of EPA and DHA, showing that n-3 PUFAs have protective effects against Type 1 diabetes by lowering islet autoimmunity in comparison with children at high risk for Type 1 diabetes who did not take n-3 PUFAs (Gorjao et al., 2009). N-3 PUFAs are of great interest to many researchers because of the various roles they play in different human disorders (Stillwell and Wassall, 2003)

1.6 Cellular Impact of N-3 PUFA

Although many studies have investigated the benefits of n-3 PUFAs toward human health, their molecular mechanisms remain unclear. N-3 PUFAs influence many biological functions, including membrane structure, ion channels, and changes in biosynthesis of eicosanoids (Arnold et al., 2010a).

1.6.1 Cell membrane

The biological properties of cell membranes are important in maintaining cellular homeostasis and function. As such, alterations to the cell membrane fatty acid composition will significantly impact cell function. The biological membrane contributes to many physical properties of the cell, such as ion permeability and elasticity (Kang, 2007; Gorjao et al., 2009). The length and number of double bonds of the acyl chain structure of fatty acids impacts the function of membrane proteins and enzymes. The structure of the fatty acid also affects the organization of the cell membrane, which has a significant influence on its physiological function. Changes to the cell membrane stimulate the intracellular signalling pathway (Gorjao et al., 2009; Adkins and Kelley, 2010). DHA is more likely to be embedded in the plasma membrane's phospholipids due to its length and double

bounds present making the structure more flexible (Lands, 2005). Evidence demonstrates that diets rich in n-3 PUFAs will have increased phospholipid pools in the plasma membrane where DHA can be 50% more than other tissues that were not under n-3 PUFAs diet. Moreover, increased in DHA intake will impact plasma membrane concentration of DHA (Wassall and Stillwell, 2008). DHA is highly flexible and dynamic, which enables it to rotate through the phospholipid membrane. Thus, even low concentrations of DHA can have a significant impact on the membrane-associated protein system (Adkins and Kelley, 2010). Cell membrane structure and fluidity is affected by the occurrence of structures with longer acyl chain and more double bounds such as DHA. It is important to maintain the ion channel concentration gradients of cell membranes between the extracellular medium and cytoplasm. Protons must diffuse through the mitochondrial membrane in order to form ATP. Studies have shown that DHA contributes more than EPA to the selectivity of protons (Gorjao et al., 2009). Another important property affected by DHA is membrane permeability; many studies report that increased DHA levels in the plasma membrane lead to increased membrane permeability. However, the relationship between membrane complexity, structure and function is still unclear (Wassall and Stillwell, 2008).

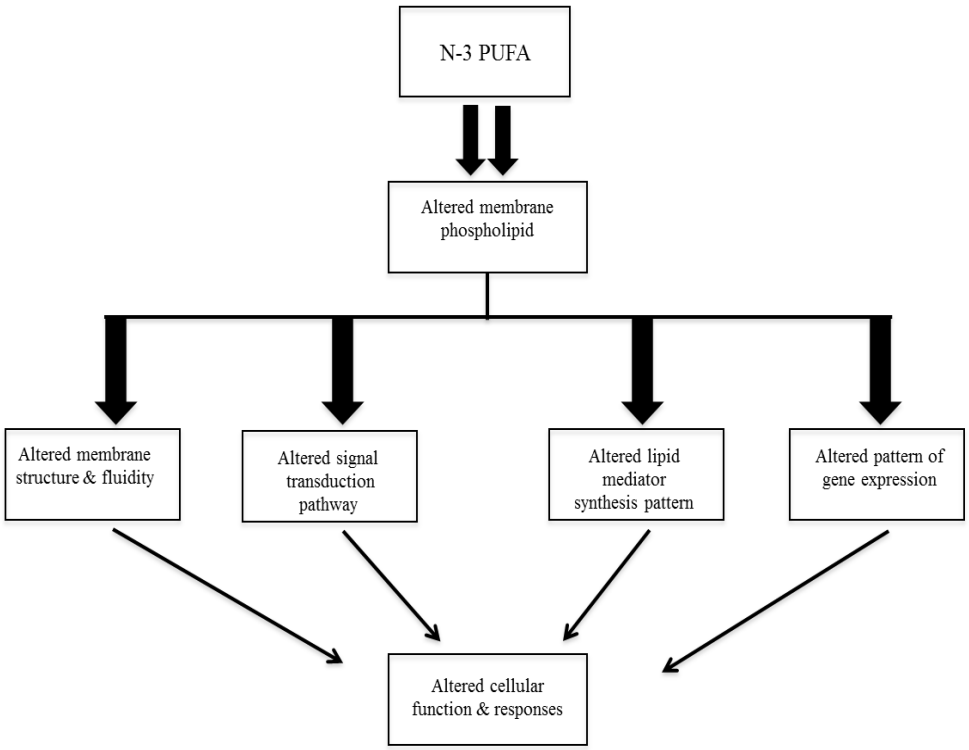


Fig.1.4 Impact of n-3 PUFAs in changing the cell membrane structure that cause changes in cellular function (adapted from (Calder and Yaqoob, 2009)).

1.6.2 Enzymatic activity

Enzymes are key components of life. Alterations in their activities will have a significant impact on cellular function. Evidence demonstrates that many enzymes are influenced by changes in dietary n-3 PUFAs (Kang, 2007). For example, all classes of phospholipases, including phospholipase C (PLC), phospholipase D (PLD) and phospholipase A_2 (PLA_2), are influenced by n-3 PUFAs (Siddiqui et al., 2008a). Phospholipase inhibition is involved in atherosclerosis and arrhythmia. Phospholipase activation contributes to improving myocardial recovery after IR. Both inhibition and activation affect calcium movement from intracellular stores (Nair et al., 1997). Evidence also suggests that free DHA or EPA is generated when PLA_2 hydrolyzes n-3 PUFAs in phospholipids (Nair et al., 1997; Siddiqui et al., 2008b).

Key enzymes such as Na^+/K^+ ATPase and Ca^{2+}/ Mg^{2++} ATPase maintain the ionic balance so that the cardiac muscle can contract and relax. A diet rich in fish oil has been shown to lead to decreases in Ca^{2+}/Mg^{2+} ATPase activity in the sarcoplasmic reticulum (Siddiqui et al., 2008a). Studies have also shown that supplementing a diet with n-3 PUFAs increases Ca^{2+}-ATPase activity in cardiomyocytes, which lowers the risk of arrhythmia by preventing Ca^{2+} accumulation after ischemia (Kang, 2007; Siddiqui et al., 2008b).

1.6.3 Ion channels

The cell membrane plays a major role in maintaining the ion concentration gradient between the cytoplasm and extracellular medium (Siddiqui et al., 2008b). Other PUFAs have less selectivity because they have shorter acyl chains and less

unsaturation compared with DHA (Gorjao et al., 2009). Intracellular Ca^{2+} plays a major role in cell contraction and vasoconstriction, and alterations in the regulation of calcium levels contribute to the development of CVD. Numerous studies have been performed to investigate the relationship between n-3 PUFAs and the plasma membrane, as well as their impact on ion channel activities and potential antiarrhythmic effects. In 1990, Kinsella et al. showed that rats fed food enriched with fish oil had higher Ca^{2+} uptake by the endoplasmic reticulum (ER), which potentially causes anti-arrhythmic effects. (Kinsella et al., 1990). Evidence has also shown that reductions in arrhythmia may be attributed to the n-3 PUFA-dependent decrease in voltage-dependent Na^{+} channel activity (Adkins and Kelley, 2010). N-3 PUFAs have a potential protective role in myocardial infarction by maintaining L-type calcium channel integrity to prevent cytosolic Ca^{2+} overload (Stillwell and Wassall, 2003; Leaf et al., 2005; Massaro et al., 2008). Generally, EPA and DHA act on ion channels via different mechanisms (Gorjao et al., 2009).

1.6.4 Mitochondria

Since the 1960s, several studies have examined the importance of PUFAs on mitochondrial function. N-3 PUFAs enhance mitochondrial function and positively affect heart function (Pepe, 2007). However, many questions about the protective mechanism of n-3 PUFAs remain unanswered (Stanley et al., 2012). Overall, investigations have shown that increased EPA and DHA levels result in changes to the phospholipid content of the mitochondrial membrane and improved ATP formation, which in turn enhance cardiac function (Duda et al.,

2009). During aging of experimental animals, the mitochondrial content of DHA was found to decrease, resulting in Ca^{2+} overload and causing the heart to become more susceptible to arrhythmias (Pepe, 2007). Similarly, changes in mitochondrial phospholipids occur as a result of consumption of n-3 PUFAs, specifically DHA; these changes could cause delays in Ca^{2+}-induced mPTP opening (Khairallah et al., 2012; Stanley et al., 2012). In hearts isolated from rats fed a diet high in n-3 PUFAs and then subjected to IR, a major shift in the DHA content of mitochondrial membrane phospholipids was observed. These phospholipids impact Ca^{2+} homeostasis, energy metabolism and oxygen consumption (Pepe, 2005; McLennan et al., 2007). Other studies have proven that enriching mitochondria phospholipids with n-3 PUFAs can help prevent apoptosis, which plays a major role in lowering the risk of cardiac diseases (Stanley et al., 2012). Most of the evidence suggests that mitochondria enriched with n-3 PUFAs help maintain a healthy functioning heart.

1.7 Cell Death

Many changes that occur in cells could be considered as mechanisms of a dead/dying cell. Cells undergo initial changes that are damaging but reversible. Eventually, they may undergo a series of irreversible changes that lead to cellular death (Kroemer et al., 2009). Several general changes in cellular features demonstrate the death of “dying cells” including fragmentation, phagocytosis, loss of plasma membrane integrity and loss of mitochondrial membrane potential (Galluzzi et al., 2007; Kroemer et al., 2009).

Many efforts have been made to classify cell death according to morphological features. The Nomenclature Committee on Cell Death (NCCD) has devised a classification system for cell death according to measurable biochemical characteristics as the assays that are quantified. Morphological classification has remained an important component of this classification system, which is attributed to the availability of microscopy in many laboratories and the familiarity of users to changes that occur in the cells (Galluzzi et al., 2012). Gross morphological changes that cells undergo during death allow for the type of death to be categorized as apoptotic, autophagic or necrotic, also known as type I, II and III cell death, respectively (Galluzzi et al., 2007; Kroemer et al., 2009). In the present study, we focus on apoptosis.

1.7.1 Apoptosis

Apoptosis, commonly referred to as programmed cell death (PCD), is a genetically programmed process that regulates pathways which activate intracellular proteins, such as Bcl-2 family proteins and cytochrome c (Ryu et al., 2010). Apoptosis can be defined as "cell death with specific morphological features" (Galluzzi et al., 2007). Key morphological criteria include cell rounding-up, plasma membrane blebbing, cytoplasm shrinkage, nuclear fragmentation, formation of "apoptotic bodies," collapse of cells into small membrane-enclosed structures and macrophage engulfment of apoptotic bodies. Two pathways, extrinsic and intrinsic, are important in activating and mediating apoptosis through the activation of caspases (Kroemer et al., 2009; Ryu et al., 2010; Whelan et al., 2010). Caspase is a peptide bond hydrolase containing an arrangement of

amino acids that are produced in inactive form and become active by proteolytic cleavage (Whelan et al., 2010; Yang et al., 2010). We discuss in detail the involvement of extrinsic and intrinsic pathways in caspase activation and indicate the cell death as a result of apoptosis.

1.7.1.1 Extrinsic pathway

The term “extrinsic apoptosis” refers to induced apoptosis by an extracellular stimulator that activates intracellular pathways, leading to cell death (Galluzzi et al., 2012). Activation of the extrinsic pathway occurs through plasma membrane receptors, known as the death receptor pathway, in response to extracellular stimulators such as stressors, cytotoxic compounds and metabolic poisons (Whelan et al., 2010). Death receptors are trans-membrane proteins that contain specific sequences of 80 amino acids called death domain (DD). Ligands bind to death receptors, which activate the corresponding death receptors on the cell membrane, for example, by binding the Fas ligand (FasL) to the Fas receptor and binding the tumor necrosis factor α (TNFα) to the TNFα receptor-1 (TNFR-1). Binding FasL triggers conformational changes in the Fas receptor that permit the DD to bind to a Fas-associated via the death domain (FADD), which has a death effector domain (DED), and activate caspase-8 (Riedl and Shi, 2004; Whelan et al., 2010; Yang et al., 2010; Harvey and Leinwand, 2011). Activating caspase-8 may directly mediate caspase-3 without the need for mitochondria; this phase is also known as caspase-dependent apoptosis (Riedl and Shi, 2004; Galluzzi et al., 2012).

1.7.1.2 Intrinsic pathway

The intrinsic pathway, also known as the mitochondrial-mediated pathway, is initiated by intracellular events that are either pathological or physiological in nature, initiating cell death via the mitochondria (Whelan et al., 2010), such as oxidative stress, hypoxia, chemical and physical toxins, will activate caspase through the enrolment of the mitochondria and loss of its integrity (Czerski and Nunez, 2004; Fumarola and Guidotti, 2004). Stimulation releases important apoptogens from the mitochondria into the cytosol that cause permeabilization of the outer membrane, such as cytochrome c. Cytochrome c is located in the mitochondrial inter-membrane space, where it participates in the electron transport chain during oxidative phosphorylation. Once released from the mitochondrial membrane, cytochrome c binds with apoptotic protease activating factor-1 (Apaf-1) in the cytosol, leading to the formation of an Apaf-1-cytochrome c complex. This complex activates caspase-9 and further causes downstream activation of caspases-7, -6 and -3 (Brenner and Mak, 2009; Whelan et al., 2010; Galluzzi et al., 2012).

The extrinsic pathway can be linked to the intrinsic pathway by mediation of caspase-8, which activates BID (the BH3-only member of the Bcl-2 family proteins) to the cleaved form tBID and mediates the release of mitochondrial proteins (Riedl and Shi, 2004). Two types of pro-apoptotic Bcl-2 protein induce death signals: Bax (Bcl-2 associated X protein) and BH3-only proteins. In normal healthy cells, Bax is present as an inactive protein in the cytosol. However, apoptotic stimuli activate Bax and BH-3-only proteins, resulting in translocation

to the mitochondria. Protein translocation enhances the release of mitochondrial apoptogens and causes permeabilization of the outer mitochondrial membrane, resulting in downstream activation of procaspases and cell death (Brenner and Mak, 2009; Whelan et al., 2010).

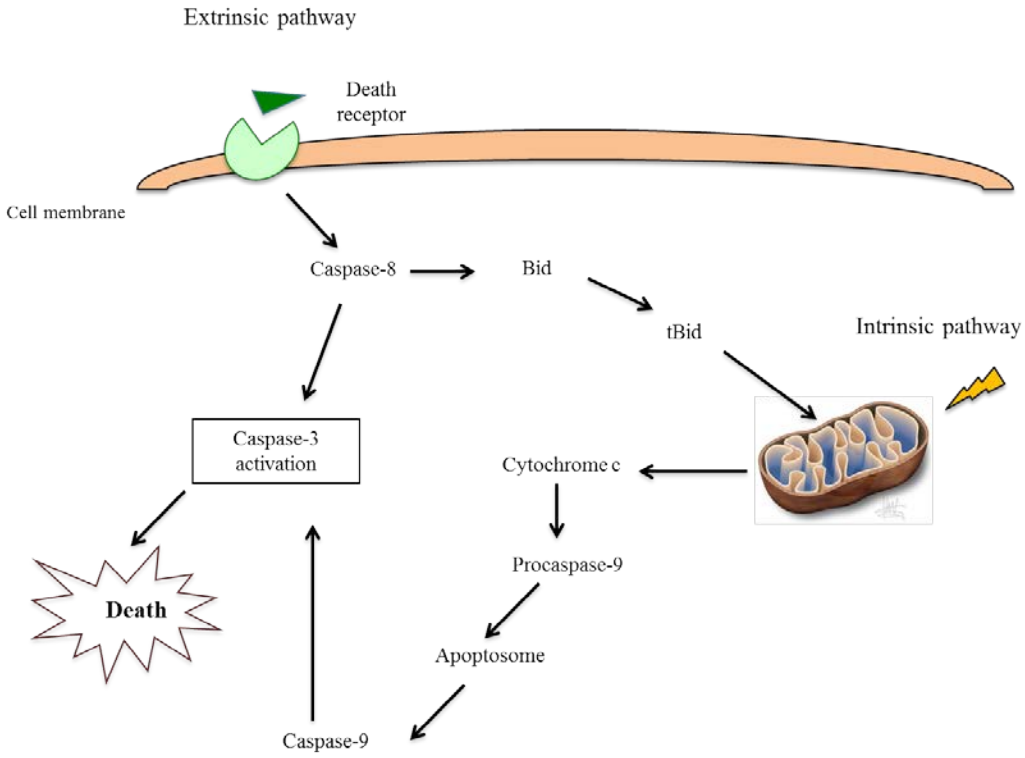


Fig.1.5 Schematic is representing mediating of apoptotic pathway (adapted from (Chiong et al., 2011)).

Chapter 2

Rationale and Hypothesis

2.1 Rationale

A significant amount of evidence shows a correlation between dietary consumption of n-3 PUFAs, specifically DHA, and lower incidence of CVD. However, the specific mechanistic role played by n-3 PUFAs in limiting cardiac damage after IR injury remains unclear. Many studies have investigated the effects of feeding animals with diets mainly containing n-3 PUFAs on cardiac functions and cellular changes. However, many inconsistencies have been found: some studies show that DHA has beneficial effects (Carroll and Roth, 2002; Xiao et al., 2008; Das and Puskas, 2009), while others fail to demonstrate that it has any significant impact on limiting cardiac damage (O'Farrell and Jackson, 1997; Billman et al., 1999; Burr et al., 2003; Huggins et al., 2009). While most of these investigations examined the effects of chronic treatment with n-3 PUFAs, however, few studies have determined how the heart responds to acute treatment with DHA. The idea behind the selection of acute treatment, which is direct dosing of the tissue or the cultured cells with specific doses and at specific times with no need to feed the animal or pretreating the cells, stems from the treatment of the heart with AA-derived CYP metabolites, EETs. As shown in our laboratory, EETs have shown improvements in recovery of heart functions after mouse hearts subjected to IR injury were isolated and directly/acutely treated with EETs (Seubert et al., 2006; Batchu et al., 2009; Chaudhary et al., 2010). In the present work, we are interested in comparing the results of direct treatment of the heart with n-6 PUFAs and n-3 PUFAs, specifically DHA. Many other studies have investigated to effects of acute DHA treatment on cells or tissues other than

heart cells (Kim et al., 2005; Xiao et al., 2008; Masi et al., 2011). In this work, we seek to determine the impact of acutely treating isolated hearts or cultured cells with DHA.

Mitochondria play a key role in maintaining proper heart function. As such, many researchers, including those in our laboratory, have focused on studying the integrity of mitochondria. The focus of the current study is to determine whether or not acute administration of DHA has any impact on improving heart function. Specifically, we were interested in studying the effect of DHA toward ischemia and reperfusion injury, and the role in maintaining mitochondrial structure and function.

2.2 Hypothesis

The global hypothesis of the present thesis is that acute administration of DHA will provide cardioprotection toward ischemia and reperfusion injury.

2.3 Thesis Aims

The goals of this thesis are:

1. To investigate effect of DHA toward cardiac functional recovery following ischemia and reperfusion injury in the Langendorff isolated heart model.
2. To investigate the role of apoptosis in DHA-mediated effects.
3. To study the impact of DHA on mitochondrial morphology and function.

Chapter 3

Material and Methods

3.1 Cell Culture

Cardiac H9c2 cells, rat myoblast cells, were purchased from ATCC (American Type Culture Collection, Manassas, VA), cultured and grown in Dulbecco's modified Eagle's medium DMEM supplemented with 10% fetal bovine serum (FBS) in antibiotic-free conditions at 37°C in a humidified atmosphere of 5% CO_2-95% air. Before the experiment, the medium was replaced with FBS free medium with 1% Penicillin-Streptomycin (Gibco, Invitrogen, Carlsbad, CA). Cells were subjected to anoxia (95% N_2/5% CO_2) followed by reoxygenation under normal growing conditions.

3.2 MTT Assay

Cell viability was measured using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. One day before the experiment, approximately 3000 H9c2 cells/well were seeded in 96-well microtitre plates. On the day of the experiment, the DMEM medium was replaced with FBS-free media containing antibiotics. Cells were treated with DHA (Cayman Chemical) and subjected to 6 or 24 h anoxia and 16 h reoxygenation. Duplicate 96-well plates were used as normoxic time-dependent controls. 5mg/mL of MTT solution were prepared in PBS (KCl, K_2HPO_4, NaCl, NaH_2PO_4) and added to the medium and incubated for 2-3 h at 37°C. The MTT solution was subsequently removed and dimethyl sulfoxide (DMSO) was added to the cells. Changes in absorbance at 590 nm were recorded in a multiplate spectrophotometer. The percentages of viable cells were determined compared to normoxic untreated cells.

3.3 Caspase-3 and -8 Activity

The activities of caspase-3 and -8 were assessed as described previously, using the specific substrates Ac-DEVD-AMC (caspase-3) and Ac-IETD-AMC (caspase-8) (Seubert et al., 2002). Briefly, cells were plated in 6-well plates in duplicate and were incubated for 48 hours or until the cells exhibited 80-85% confluence. One plate was kept in normoxic conditions and the other plate was subjected to anoxia/ reoxygenation. Cells were harvested and centrifuged for 10 min at 1500 ×g at 4℃, the supernatant was removed and the residual pellet was resuspended in 200 μL of lysis buffer (10mM Tris-Hcl, 130mM NaCl, 1% Triton X100, 10mM NaF, 10mM NaH_2PO_4, and 10mM $Na_4P_2O_7.10H_2O$). Cells were homogenized for 20-30 sec and centrifuged for 15 min at 15000 g. Enzymatic activity was assessed by incubating 20 μL of supernatant fraction with 180 μL of reaction buffer (20 mM HEPES, pH 7.4, 10% glycerol, 2 mM dithiothreitol, and 20 μM Ac-DEVD-AMC or Ac-IETD-AMC) in the dark at 37°C for 1 h. The fluorescence was monitored at wavelengths of 380 (excitation) and 460 nm (emission). The activity was calculated by using a linear standard curve found with AMC.

3.4 Immunocytochemistry

Cells cultured on sterile coverslips were treated with DHA (0, 10 or 100 μM) for 3 or 6 hours. Mitochondrial integrity were assessed by staining the cells with 100 nM of Mito Tracker Orange (CM-H_2TMRos), which was applied 45 min prior to cell fixation. Cells were fixed for 10 min using 4% paraformaldehyde (PFA) and washed three times with PBS. Coverslips with fixed cells were stored

in PBS with 0.1% NaN_3 at 4°C. Cells were permeabilized by incubating with 0.5% Triton X-100 for 10 min and blocked using 10% BSA and 0.3M glycine with 10% donkey serum for 1 hour at 25°C. Primary antibody (rabbit-cleaved caspase-3) (Cell Signaling Technology Inc., Danvers, MA, USA) at concentration 1:200 was incubated overnight at 4°C. Secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG) (Molecular Probes, Invitrogen Detection Technologies, ON, CA) at a concentration of 1:200 was incubated for 1 hour at room temperature. Images were taken by using fluorescence microscopy at wavelengths of 488 (excitation) and 590 nm (emission) for cleaved caspase-3 and wavelengths of 554 (excitation) and 576 nm (emission) for mito Tracker orange.

3.5 Mitochondrial Membrane Potential Assessment

Changes in mitochondrial morphology and activities were assessed as previously described (Zhang et al., 2009; El-Sikhry et al., 2011). Briefly, H9c2 cells were plated in 35mm glass bottom dishes and incubated until they exhibited 80-85% confluence. One hour prior to treatment, cells were loaded with 100 nM tetramethylrhodamine ethyl ester (TMRE) and 1mM Hoechst 33342 trihydrochloride. TMRE is used to reflect the mitochondrial function by polarizing its membrane and Hoechst 33342 is used to stain the cell nucleus. The cells were treated with 100 μM DHA and images taken with a Zeiss Axio Observer Z1 inverted epifluorescence microscope. The cells were maintained at 37°C and 5% CO_2 throughout the experiment.

3.6 Western Blotting

Cells were first lysed in a 100 μL lysis buffer (130mM NaCl, 10mM NaF, 10mM NaH_2PO_4, 10mM $Na_4P_2O_7$*$10H_2O$, 1% Triton X100, 10 mM Tris-HCl, pH7.4) in the presence of a 5 μL/mL protease inhibitor cocktail, homogenized and then centrifuged at 1500 ×g for 15 min at 4 °C. Protein content was measured in supernatant fractions using a Bradford protein assay kit (BioRad Laboratories, Canada). SDS-poly-acrylamide gel electrophoresis was performed using 25 μg protein and transferred to nitrocellulose membranes. The blots were incubated with a primary antibody (mouse monoclonal Opa 1 and anti-cytochrome c 1:1000) overnight at 4°C and then washed three times using a washing buffer. The secondary antibody anti-mouse IgG 1:10000 was incubated at room temperature for 2-3 hours and then rinsed three times with washing buffer. Chemiuminscence solution (ECL Plus; Amersham Biosciences, PQ, Canada) was used to detect the signals.

3.7 Cytochrome c Release

Cytochrome c released from the mitochondria to cytosol was assessed as previously described (Seubert et al., 2002). Briefly, following treatment, H9c2 cells were washed with PBS and harvested by using trypsin. The cells were centrifuged for 10 min at 1500 ×g. PBS was removed and the cells were incubated with a digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM $NaHPO_4$, 250 mM sucrose, 5 μg/mL digitonin and 5 μL/mL protease inhibitor cocktail) on ice for 5 min to the perforate plasma membranes. The suspended cells were centrifuged at 14,000 ×g for 10 min at 4°C to separate out the supernatant

(cytosol) and heavy membrane fractions (crude mitochondrial fraction). The heavy membrane fractions were suspended in Tris-HCl buffer (25 mM Tris-HCl, pH 8.0, and 0.1 % Triton X-100).

3.8 Animals and Isolated-Perfused Hearts

All experiments used C57bL/6 male mice aged 3–5 months weighing 22–33 g. The mice were treated in accordance with guidelines from Health Science Laboratory Animal Services (HSLAS), University of Alberta. C57BL6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ).

Hearts were isolated and perfused in the Langendorff mode as previously described (Seubert et al., 2006; Chaudhary et al., 2009; Chaudhary et al., 2010). Briefly, the animals were anaesthetized, the chests were opened and the hearts isolated and placed in a cold Krebs-Hensleit buffer (NaCl, Glucuse, $NaHCO_3$, Na Pyrivate, CaCl stock and salt stock). Hearts were cannulated via the aorta and perfused in the Langendorff mode. A balloon was placed in a small hole in the left ventricular wall and connected to an electromanometer to record the left ventricular pressure (LVP). The heart pressure was kept constant (90-120 cmH2O) and equilibrated with a mixture of 95%O_2/5%CO_2 at 37°C. Hearts were perfused with the buffer for 40 minutes of stabilization and then subjected to 30 minutes of global no-flow ischemia, followed by 40 minutes of reperfusion. Different treatments (DHA 0, 10, 50, 100 µM) were added when the baseline was stable (20 min). Throughout the experimental protocol, all hearts were maintained at 37°C in a thermostatically controlled glass chamber. The percentage of

preischemic of the left ventricular developed pressure (LVDP) was calculated by taking the recovery of contractile of LVDP at the end point of reperfusion.

3.9 Statistical Analysis

To analyze the data and determine the statistical significance, we used t-test for two groups comparisons and one-way ANOVA (Duncan's test) for multiple groups comparisons. Values were expressed as mean ±SEM and considered significantly different if $P<0.05$.

Chapter 4

Results

4.1 Reduction in Cell Viability

H9c2 cells were plated in 96 well-plates. The medium was changed to serum free and treated with different doses of DHA (0, 1, 5, 10, 50 and 100 µM). Cells were subjected to either short (6 hours) or long (24 hours) periods of anoxia followed by 16 hours reoxygenation. There were significant reductions in the percentage of viable cells following treatment with 50 and 100 µM DHA in all groups whether the cells were in the normoxic or anoxic-reoxygenation condition. No significant cell death was observed at the lower doses (1, 5 and 10 µM DHA) (Fig.4.1 A & B). However, when the anoxic condition was increased to 24 hours/16 hours reoxygenation (Fig.4.2), there were significant reductions in the percentage of viable cells for H9c2 cells that were treated with all the DHA groups (Fig. 4.2 B). The DHA metabolite, EDP, had no significant effect when H9c2 cells were treated with (0, 0.1, 0.5 and 1 µM EDP) and subjected to 24 h anoxia/16 h reoxygenation (Fig.4.3 A and B). The 2 µM EDP showed significant decrease in the cell viability in anoxic/reoxygenation condition (Fig.4.3 B) but no differences in the normoxic condition (Fig.4.3 A).

These results suggest that low concentrations of DHA (>10 µM) have no significant effect under normoxic conditions, but that increased cell stress stemming from anoxia/reoxygenation results in enhanced cell death by DHA. High DHA concentrations (50 and 100 µM) are toxic to H9c2 cells under both normoxic and anoxic/reoxygenation conditions.

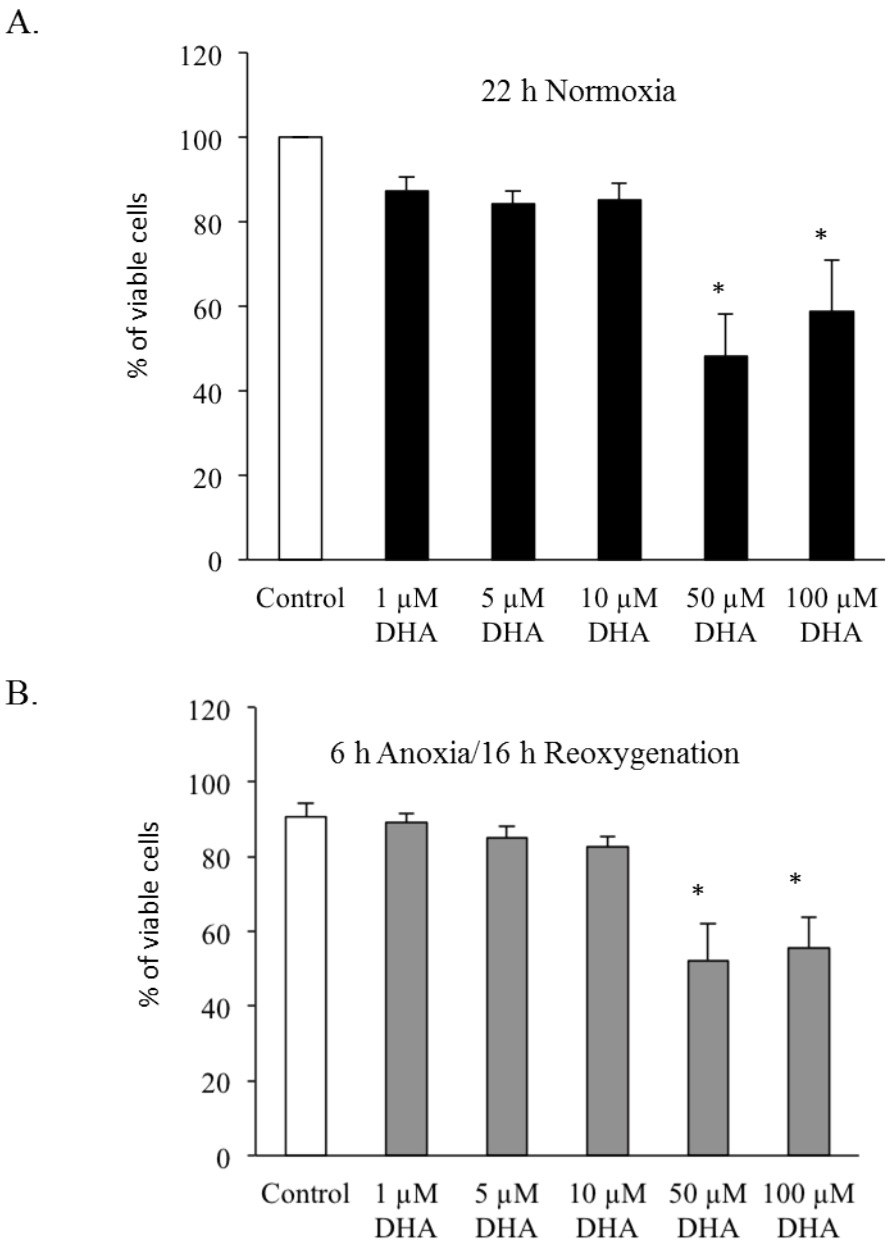
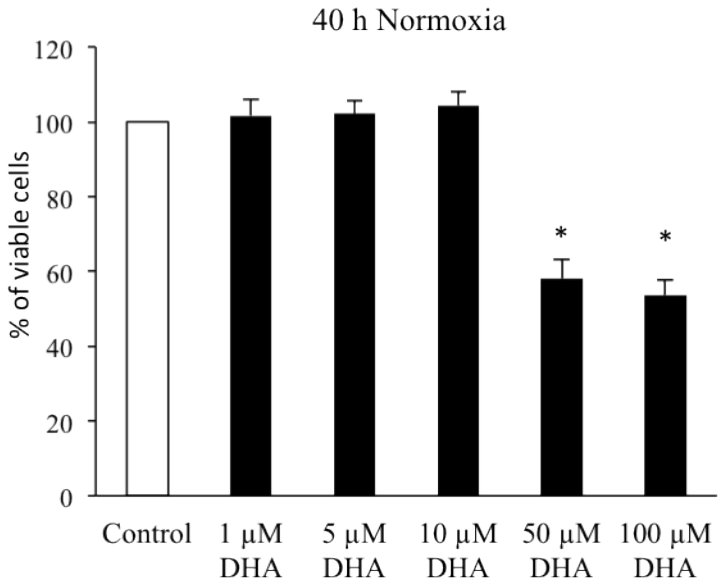


Fig. 4.1 Cell viability: histogram representing the percentage of viable cells after H9c2 cells being treated with DHA (0, 1, 5, 10, 50 and 100 µM). A) H9c2 cells were treated with DHA and remained in a normoxic condition for 22 h (corresponding to anoxic/reoxygenation plate). B) H9c2 cells were treated with DHA and subjected to 6 h anoxia followed by 16 h reoxygenation. Values represent the mean ± SEM, n=4; *, P <0.05 compared with vehicle control.

A.



B.

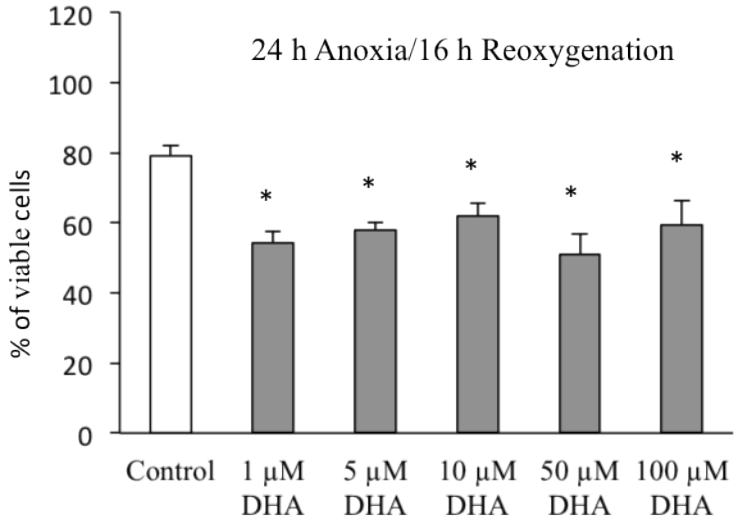


Fig. 4.2 Cell viability: histogram representing the percentage of viable cells after being treated with DHA (0, 1, 5, 10, 50 and 100 µM). A) H9c2 cells were treated with DHA and remained in a normoxic condition for 40 h (corresponding to anoxic/reoxygenation plate). B) H9c2 cells were treated with DHA and subjected to 24 h anoxia followed by 16 h reoxygenation. Values represent the mean ± SEM, n=4; *, P <0.05 compared with vehicle control.

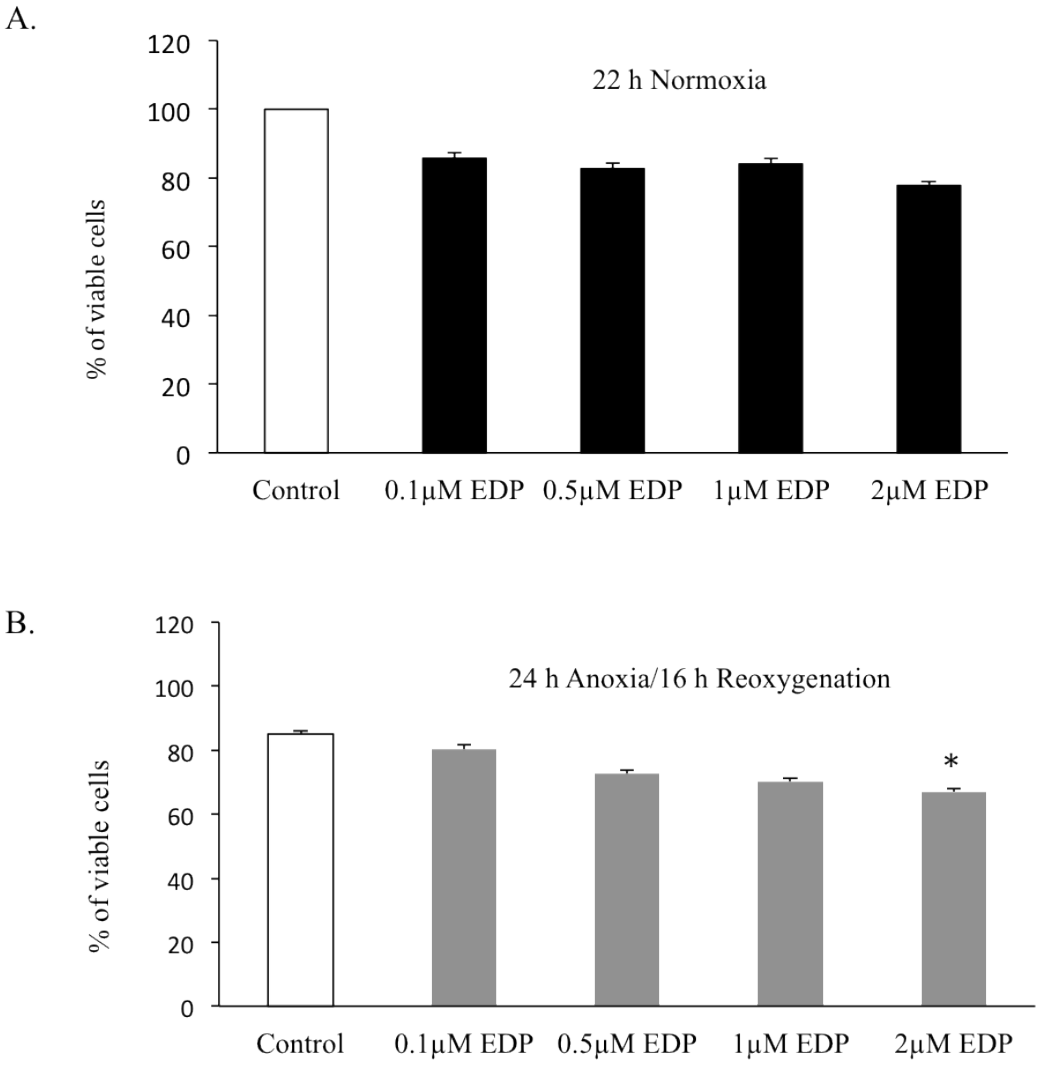


Fig. 4.3 Cell viability: histogram representing the percentage of viable cells after being treated with EDP (0, 0.1, 0.5, 1, and 2 μM). A) H9c2 cells were treated with EDPs and remained in a normoxic condition for 40 h (corresponding to anoxic/reoxygenation plate). B) H9c2 cells were treated with EDPs and subjected to 24 h anoxia followed by 16 h reoxygenation. Values represent the mean ± SEM, n=3; *, P <0.05 compared with vehicle control.

4.2 Increases in Caspase-3 and -8 Activity

Significant cell death was seen in H9c2 cells treated with 10 and 100 µM DHA after 24 hours of anoxia, as such this time point was utilized in subsequent experiments. Microscopic observations demonstrated obvious detachment (dead cells) and rounding of cells from the bottom of the plate, suggesting an apoptotic cell death (Fig 4.4). A low concentration of DHA (10 µM DHA) did not show any significant cell death under normoxic conditions in cell viability (Fig.4.1 and 4.6), but significant increases in caspase-8 and -3 activities were observed (Fig.4.5 and 4.6). This observation could be explained by a minor toxic effect of low DHA concentration that was sufficient to activate the caspases' activity but not sufficient to cause a major or quantified cell death. Cells treated with high concentrations of DHA (100 µM DHA) had significant increases in both caspase-8 and -3 activities, in the presence or absence of a stressor (anoxia/reoxygenation) (Fig. 4.5 and 4.6).

To further validate the activation of caspase-3 activity, H9c2 cells were treated with DHA for 3 and 6 hours under normoxic conditions to observe the initiation of cleaved caspase-3 using immunocytochemistry (Fig.4.7). Cells with activated caspase-3 were not detected following 3 hours of treatment with 10 µM DHA, but cleaved caspase-3 was detected at 6 hours (Fig.4.7). A strong activation of caspase-3 was detected as early as 3 hours following incubation with 100 µM DHA with a stronger signal observed at 6 hours (Fig.4.7 A). Quantification of the percentage of positive cleaved caspase-3 cells treated with DHA were calculated and compared to control (Fig.4.7 B), in which 100 µM DHA treatment resulted in

a statistically significant increase. These results confirm the significant increase in caspase-3 enzymatic activity that was shown in (Fig.4.6). We can conclude from these observations that 10 and 100 μM DHA initiates caspase-3 activation early (3 or 6 hours), confirming an apoptotic cell death.

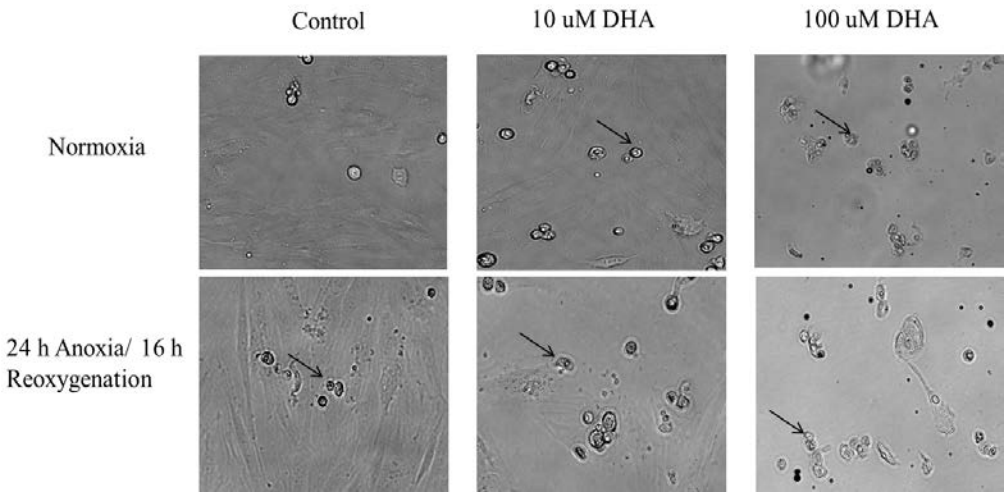


Fig 4.4 Representative phase contrast images demonstrating cellular morphological changes following treatment of 10 and 100 µM DHA and subjected to 24 h anoxia/ 16 h reoxygenation in H9c2 cells. Arrows highlight cellular rounding and detachment reflecting apoptotic cell death. Images are from a single representative experiment, which was repeated 4 times.

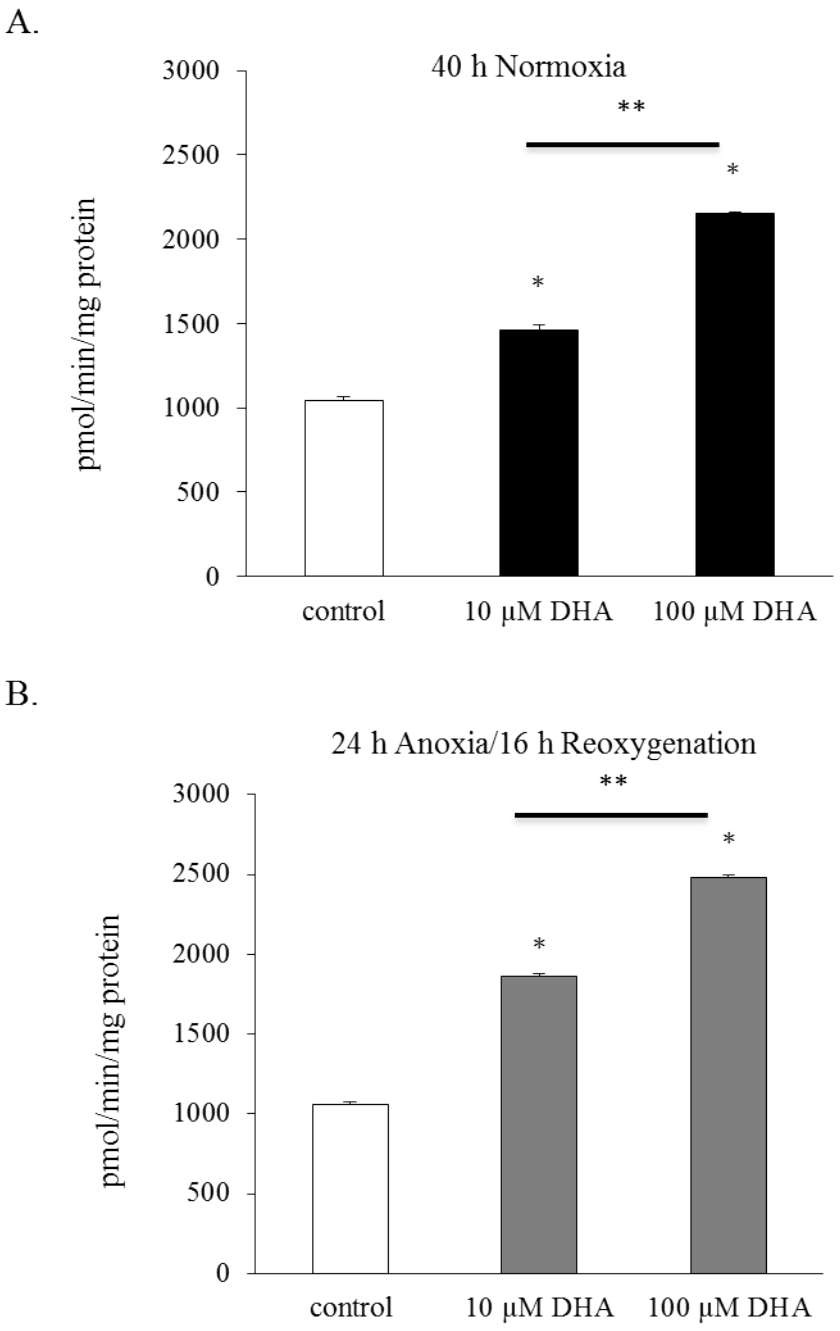


Fig.4.5 Activation of caspase-8. A) Caspase-8 activity in H9c2 cells after incubation with 0, 10 and 100 μM DHA, and remained for 40 h in a normoxic condition. B) Caspase-8 activity in H9c2 cells after incubation with 0, 10 and 100 μM DHA and subjected to 24 h Anoxia/16 h reoxygenation. Values represent the mean ± SEM, n=3; *, *P* <0.05 compared with vehicle control, ** *P* <0.05, 10 vs. 100 μM DHA.

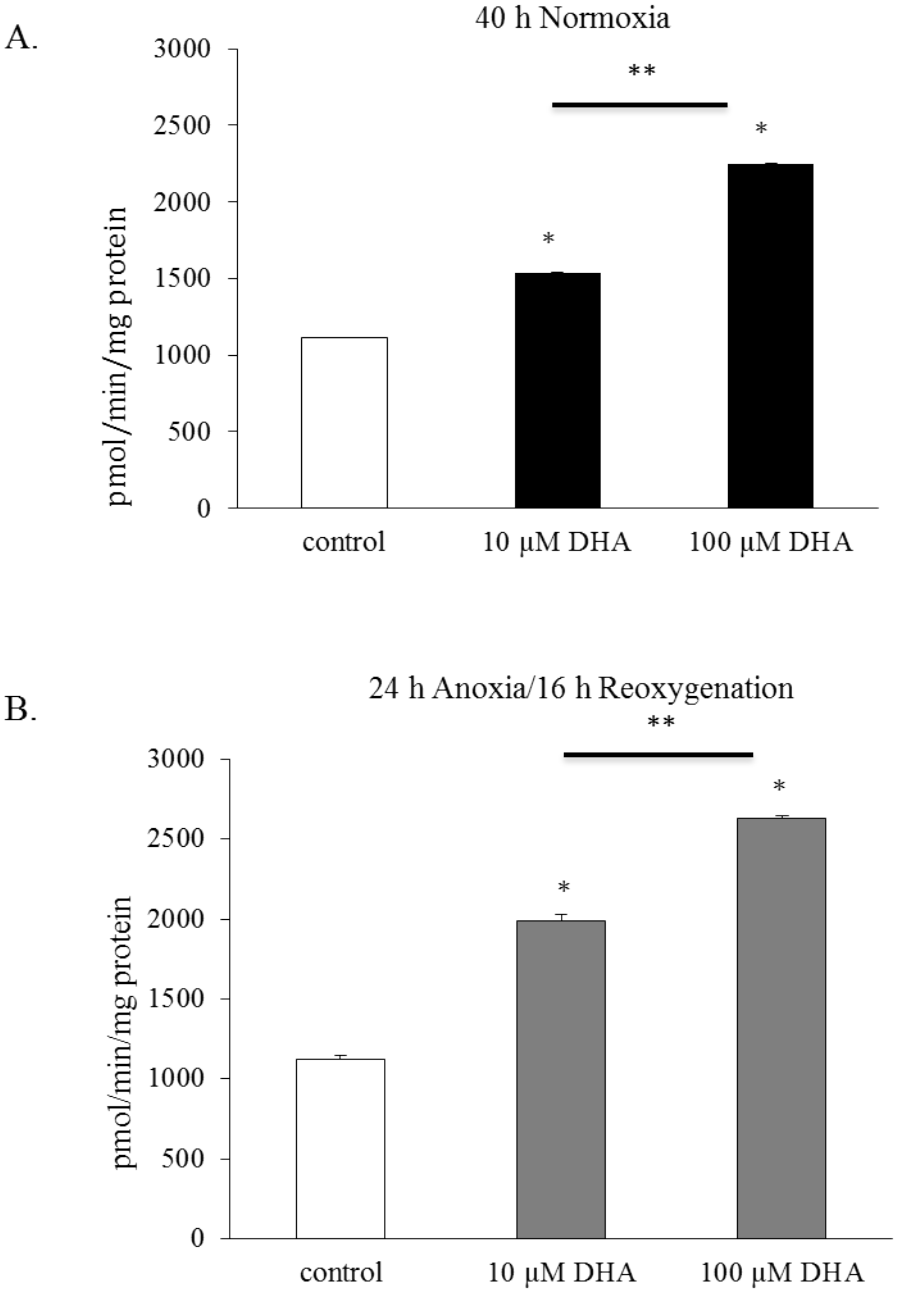


Fig.4.6 Activation of caspase-3. A) Caspase-3 activity in H9c2 cells after incubation with 0, 10 and 100 μM DHA and remained for 40 h in a normoxic condition. B) Caspase-3 activity in H9c2 cells after incubation with 0, 10 and 100 μM DHA and subjected to 24 h Anoxia/16 h reoxygenation. Values represent the mean ± SEM, n=3; *, P <0.05 compared with vehicle control, ** P <0.05, 10 vs. 100 μM DHA.

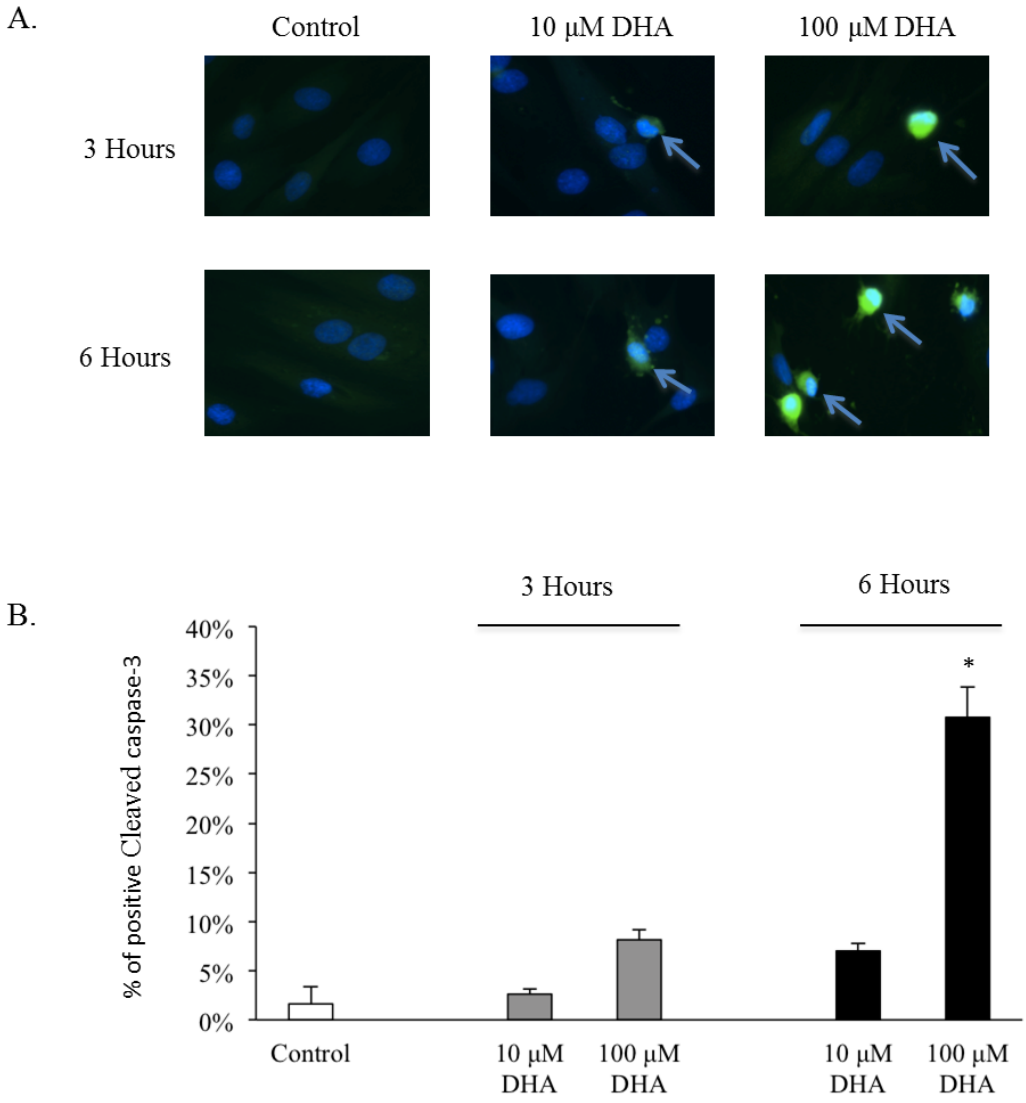


Fig.4.7 Apoptotic response as a result of acute administration of 0, 10 and 100 µM DHA on H9c2 cells. A) Representative IHC images stained with cleaved caspase-3 (green) and DAPI (nucleus – blue) in H9c2 cells treated with 0, 10 and 100 µM DHA for 3 and 6 h in a normoxic condition. B) Histogram representing the percentage of activated cleaved caspase-3. Values represent the mean ± SEM, n=3; *, P <0.05 compared with vehicle control.

4.3 Changes in Cellular Mitochondrial Activities

Mitochondria have an essential function in maintaining the homeostasis of cell survival and cell death. They have an important role in initiating apoptosis (programed cell death), for example mitochondrial damage or dysfunction will result in the release of cytochrome c into the cytosol leading to activation of caspases (Landes and Martinou, 2011). Therefore, in this study we emphasized on the role of mitochondrial function.

Real-time imaging was used to determine any changes in mitochondrial morphology and membrane potential by loading H9c2 cells with TMRE. Cells that were treated with 100 μM DHA had dramatic mitochondrial fragmentation and dysfunction. DHA-treated cells had mitochondria that were shorter and more rounded than the healthy long mitochondria of the untreated control cells. DHA caused dissipation of fluorescence, which indicated depolarization of the mitochondrial membrane potential (Fig.4.8). In addition, there was a significant change in mitochondrial morphology following treatment with DHA (Fig.4.9 and 4.10). This data suggests that DHA causes significant mitochondrial damage, which correlates with enhanced cell death.

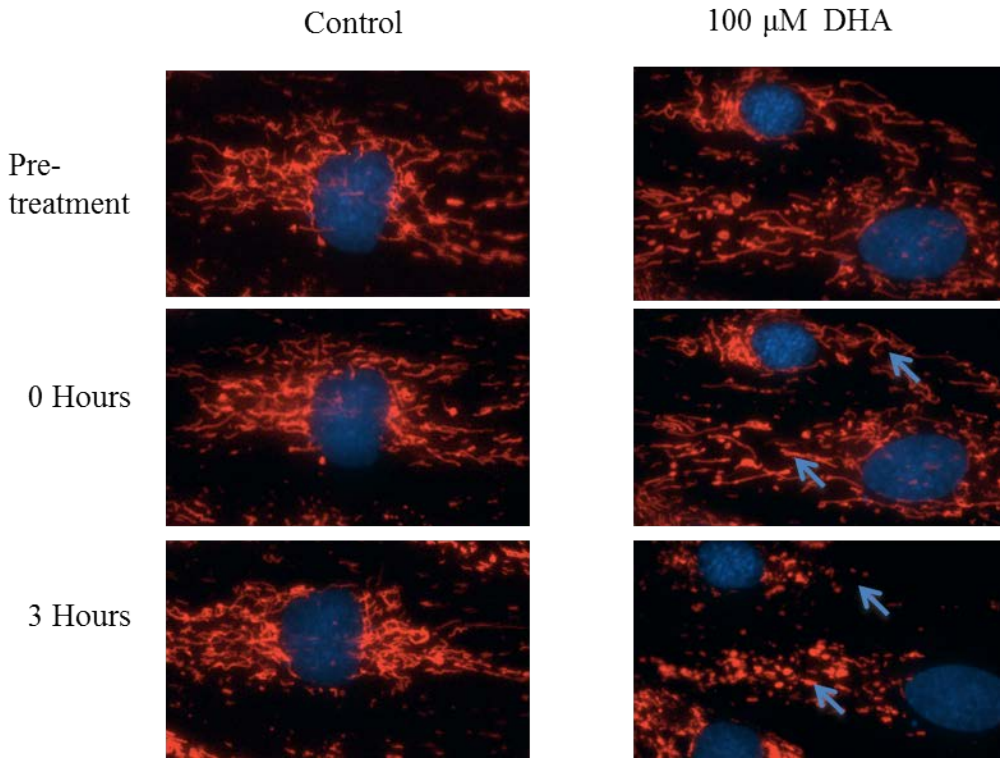


Fig.4.8 Assessment of mitochondrial morphology in H9c2 cells. Representative images showing H9c2 cells before adding the treatment (first row), 0 h after adding the treatment (middle row) and 3 h after adding the treatment (last row). The mitochondrial morphology, filamentous and tubular shape, of the control cells remained unaltered during this time period. In contrast, DHA-treated cells showed significant mitochondrial fragmentation and changes in mitochondrial morphology.

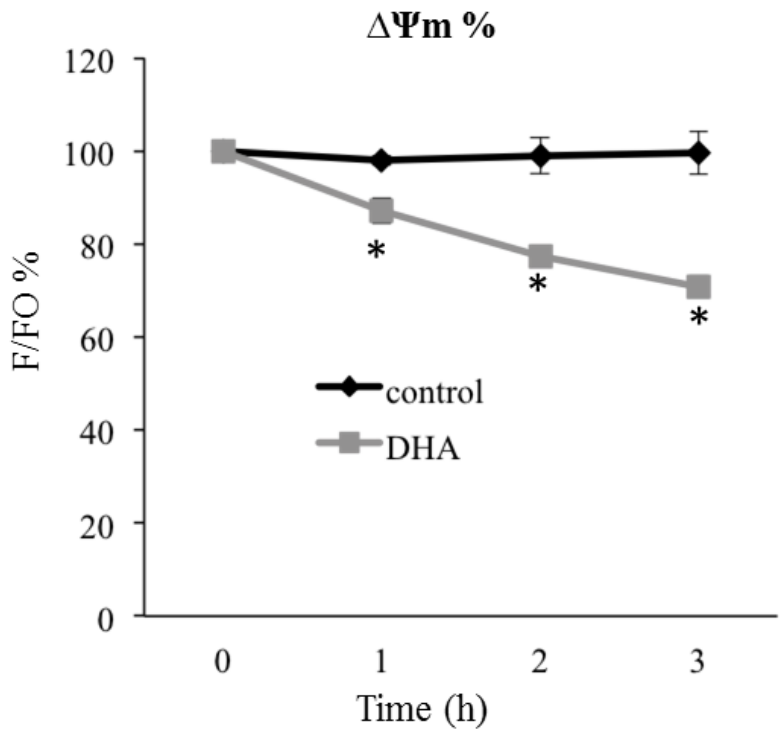


Fig.4.9 Percentage of tetramethylrhodamine ethyl ester (TMRE) fluorescence lost in H9c2 cells after treatment with 100 µM DHA. Values represent the mean ± SEM, n=3; *, *P* <0.05 compared with control.

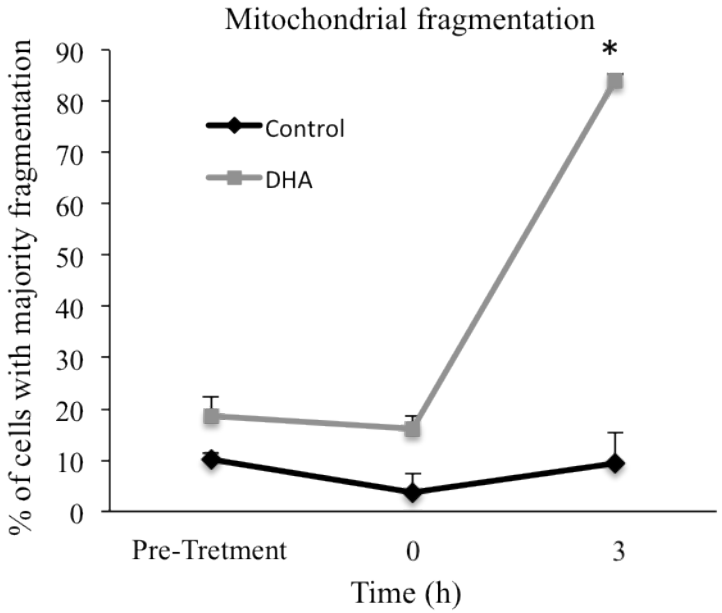


Fig.4.10 Percentage of cells with fragmentation present in individual cells relative to total mitochondria. Values represent the mean ± SEM, n=3; *, *P* <0.05 compared with control.

Mito Tracker Orange was used to confirm the impact of DHA on the mitochondria. Cells were treated for 3 or 6 hours under normoxic conditions and cellular mitochondria were assessed using immunocytochemistry (Fig.4.11). Mito Tracker Orange was added 45 min prior to the end of treatment and cell fixation. Mitochondria appeared tubular and retained strong fluorescent signals in the untreated cells. A loss of fluorescence was observed in a time- and concentration-dependent manner; with activity gradually decreasing for 100 µM DHA treated cells at 3 to 6 hours. A rapid loss of fluorescence occurred in cells treated with 100 µM DHA. The loss was significant within the first 3 hours and complete by 6 hours. These data support previous findings (Fig.4.8) demonstrating a loss of mitochondrial membrane potential following DHA treatment.

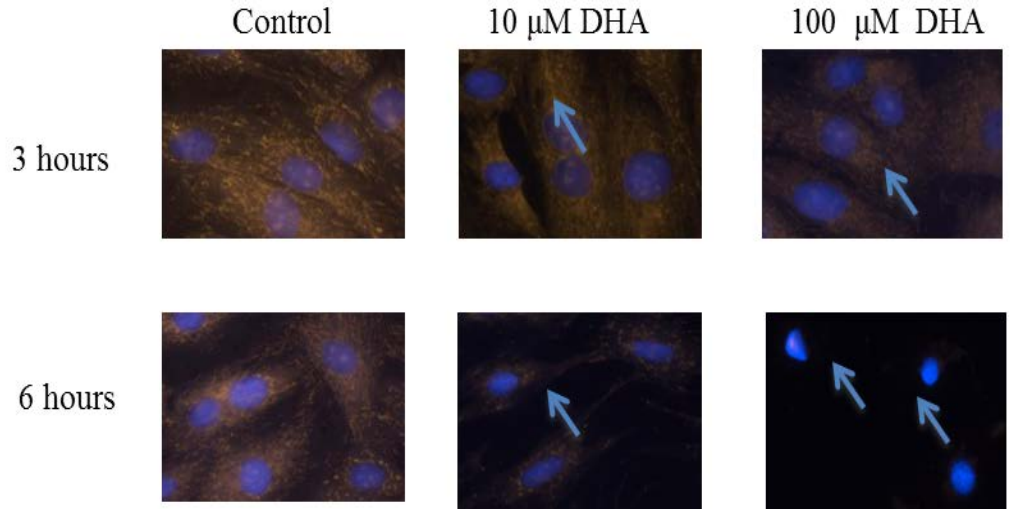


Fig.4.11 Immunocytochemistry staining the mitochondria with Mito Tracker Orange. Representative images showing loss of mitochondria membrane potential following treatment of H9c2 cells with 0, 10 and 100 µM DHA.

In order to further determine the role of mitochondria in the cellular apoptotic response we observed with DHA, we assessed the release of cytcohrome c following treatment. H9c2 cells were treated with DHA for 6 hours. The release of cytochrome c was determined by western blot analysis. A concentration-dependent increase in the expression of cytochrome c was observed in cytosolic fractions following DHA treatment (Fig.4.12). No expression of prohibitin (mitochondrial marker) was detected in the cytosol. Activation of caspases-3 and -8 and the cytochrome c release through mitochondria are evidence that DHA enrolment is active in the intrinsic and extrinsic apoptotic pathways that mediate the mitochondria.

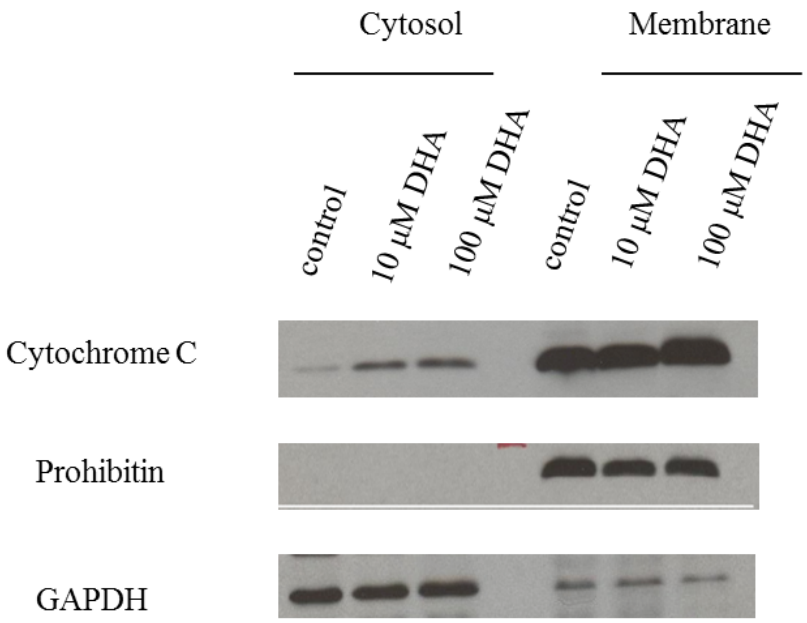


Fig.4.12 Mitochondrial cytochrome c release in H9c2 cells after incubating for 6 h with DHA (0, 10 and 100 μM). Data are from a single representative experiment, which was repeated three times.

Opa 1 is a crucial protein, located in the mitochondria's inner membrane. Opa 1 has an essential role in maintaining mitochondrial cristae structure (Sheridan and Martin, 2010). Reduction in Opa1 expression from the long to short isoform is associated with increased fragmentation and may trigger cell death (Chen et al., 2009). Thus, together with our results demonstrating increased mitochondrial fragmentation (Fig.4.13 and 4.14), we assessed the effect of DHA on the degradation of mitochondrial Opa1. H9c2 cells were treated with different concentrations of DHA for 6 or 24 hours. No significant changes in Opa1 expression were observed following 6 hours of treatment (Fig.4.13). However, a concentration-dependent decrease in Opa1 expression was observed following 24 hours (Fig.4.14).

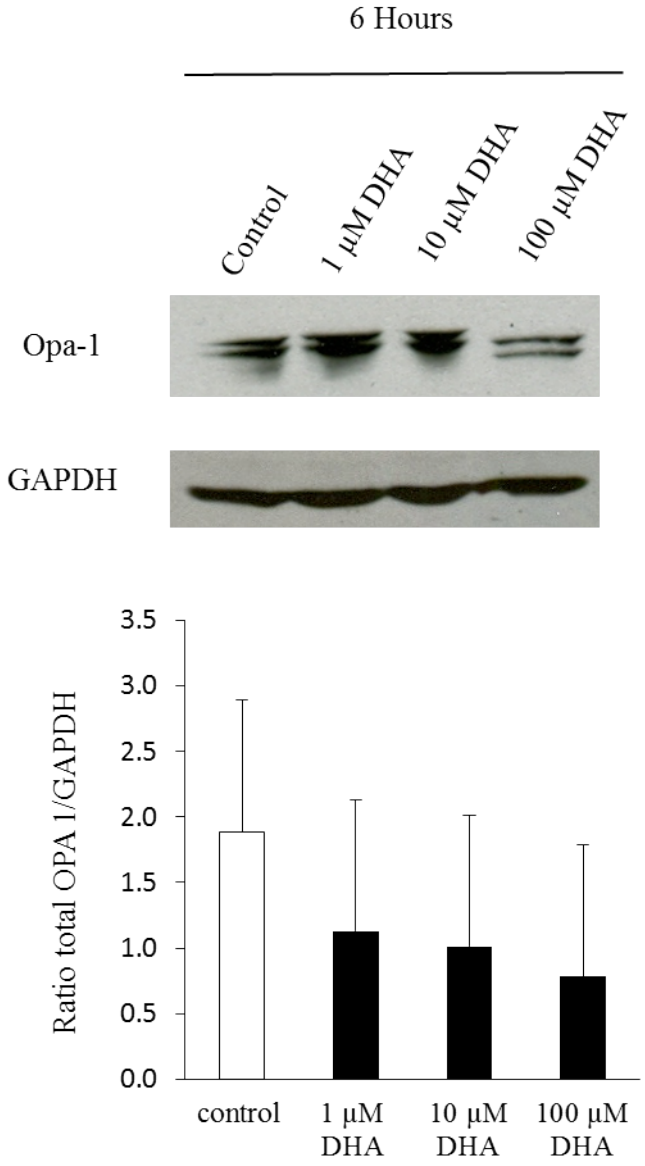


Fig.4.13 DHA induces mitochondrial damage. Immunoblot showing Opa 1 expression from H9c2 cells treated with DHA for 6 h. Data have been repeated three times.

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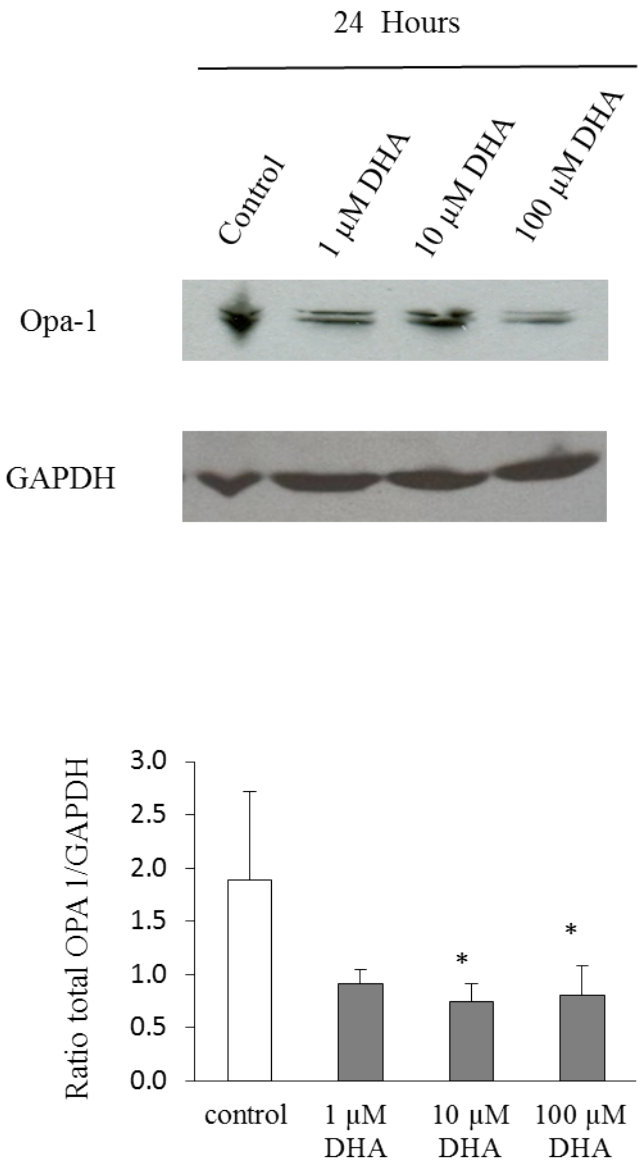

Fig.4.14 DHA induces mitochondrial damage. Immunoblot showing Opa 1 expresion from H9c2 cells treated with DHA for 24 h. Values represent the mean ± SEM, n=3; *, *P* <0.05 compared with normoxic untreated control.

4.4 Cardiac Function

To determine whether DHA protected against damage caused by ischemia-reperfusion injury, hearts from C57BL/6 mice were perfused with different concentrations of DHA (0, 10, 50 or 100 µM). All hearts were first perfused for 20 minutes without treatment to obtain baseline data, after which DHA was added. No significant effects on cardiac function were observed in hearts perfused with 10 µM DHA compared to the control (Table 1). Perfusing hearts with either 50 or 100 µM DHA at 20 min baseline (B20) resulted in significant decreases in LVDP and diastolic (dP/dt min) and systolic rates (dP/dt max) in preischemic levels compared to the control groups (Table 1). In addition, perfusion with 50 or 100 µM DHA decreased the preischemic heart rate (Table 1). Hearts perfused with 10 µM did not show any significant improvement in postischemic LVDP compared to vehicle-treated animals (Fig. 4.15.). Moreover, hearts perfused with 50 or 100 µM DHA showed a significant decrease in the percentage of functional recovery compared to hearts in the control group.

A caspase-3 assay has been assessed in heart tissue treated with DHA. We did not find any significant changes in the caspase-3 activity after treating the heart with DHA and subjecting it to 30 min ischemia and 40 min reperfusion (Fig.4.16). This finding suggests that the reduction in heart function could involve a pathway other than apoptosis. The changes in the heart function could be related to the impact of the ion channels as a result of DHA treatment.

As previously shown, DHA-derived epoxy metabolites of CYP, EDP, could play a role in cardioprotection (VanRollins et al., 1989; Ye et al., 2002;

Arnold et al., 2010a). We have hypothesized that EDP could help to prevent injury from ischemia/reperfusion. Isolated mice hearts were perfused with different EDP concentrations (0.1, 0.5 and 1 μM) and subjected to ischemia/reperfusion injury. Perfused hearts with EDPs did not show any significant changes in heart functional recovery after ischemia and reperfusion injury (Fig.4.17). Regarding the other cardiac function, there were no significant changes in the heart rate, or in the LVDP and diastolic (dP/dt min) and systolic rates (dP/dt max) in preischemic and postischemic levels compared to the control groups (Table 2). Our results indicate that EDP does not play a role in preventing the damage that occurs after an ischemia/reperfusion injury.

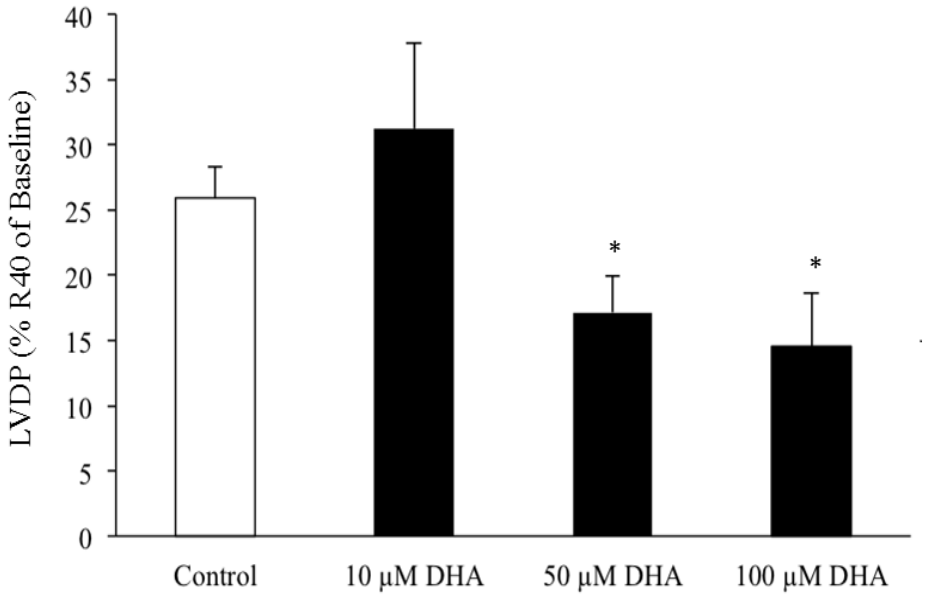

Fig.4.15 LVDP recovery at 40 min of reperfusion as percentage of baseline. Hearts were perfused with 0, 10, 50, and 100 μM DHA and subject the heart to 30 min ischemia followed by 40 min reperfusion. Values represent the mean ± SEM, n=4-8 hearts; *, P <0.05 compared with control.

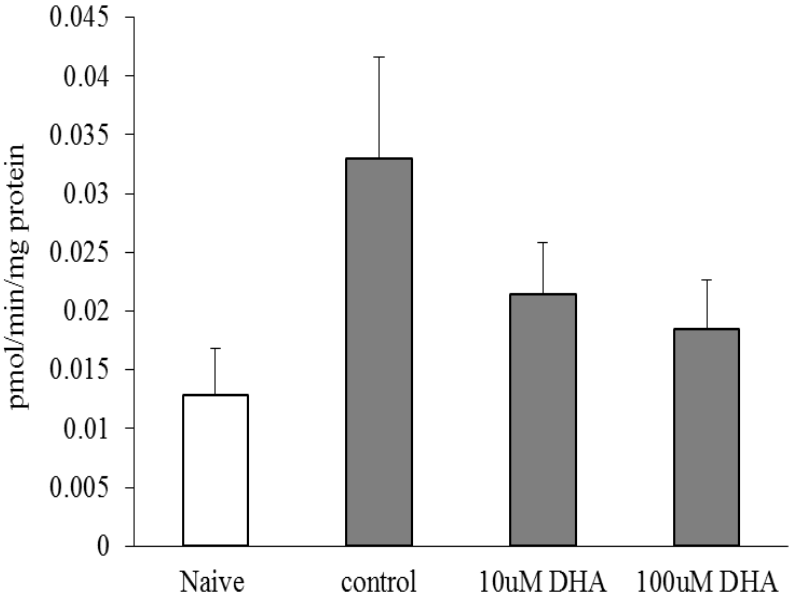

Fig.4.16 Caspase-3 activity of heart tissue isolated and treated with 0, 10 and 100 μM DHA. The heart was subjected to 30 min ischemia followed by 40 min reperfusion.

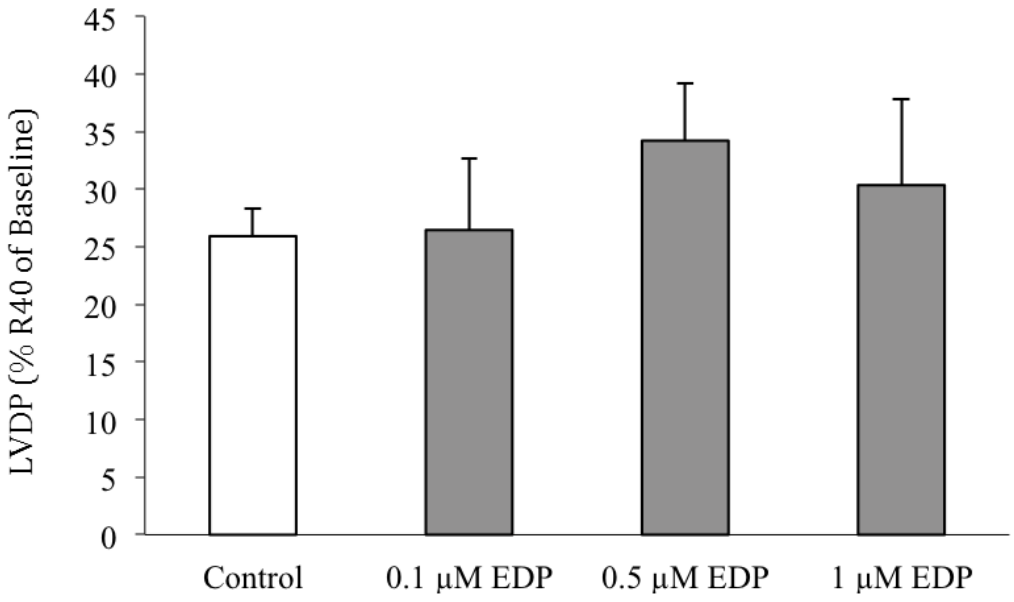

Fig.4.17 LVDP recovery at 40 min of reperfusion as percentage of baseline. Hearts were perfused with 0, 0.1, 0.5, and 1 μM EDP and subject the heart to 30 min ischemia followed by 40 min reperfusion.

Baseline Before Treatment	**Control**	**10 μM DHA**	**50 μM DHA**	**100 μM DHA**
LVDP (cmH_2O)	97.4±8.4	103.5±17.8	103.5±18.3	123.6±23.8
Rate of Contraction (cmH_2O/msec)	1955.1±458.8	2853.5±600.4	3291.9±622.5	3150.9±773.1
Rate of Relaxation (cmH_2O/msec)	-1302±381.8	-2362.6±527.3	-2394.1±458.8	-2527.5±393.1
Heart Rate (beat/min)	184.6±29.5	245.2±34.5	275.8±27.2	270.9±45.6
After Treatment and Pre-ischemia				
LVDP (cmH_2O)	99.6±6	102.0±24.3	68.9±6.2*	29.5±12.8*
Rate of Contraction (cmH_2O/msec)	3051.9±916.7	2984.8±691.1	1843.9±248.6*	1443.9±598.9*
Rate of Relaxation (cmH_2O/msec)	-1750.7±449.4	-1550.9±555.9	-1251.1±125.0	-1001.9±360.3
Heart Rate (beat/min)	208.0±35.9	265.6±17.8	187.1±25.1	116.6±27.1*
Post-ischemia				
LVDP (cmH_2O)	27.1±3.7	27.1±3.9	15.5±3.1*	19.4±4.9
Rate of Contraction (cmH_2O/msec)	966.2±355.9	1021.5±231.5	313.0±116.9*	604.3±146.9
Rate of Relaxation (cmH_2O/msec)	-815.4±302.3	-859.4±188.1	-328.6±71.6*	-492.1±79.1*
Heart Rate (beat/min)	296.7±47.4	219.4±19.4	219.8±52.2	63.4±18.0*

Table 4.1 The impact of different DHA concentrations on cardiac function

Baseline Before Treatment	**Control**	**0.1 μM EDP**	**0.5 μM EDP**	**1 μM EDP**
LVDP (cmH_2O)	97.4±8.4	124.8±21.4	107.4±13.1	103.2±11.6
Rate of Contraction (cmH_2O/msec)	1955.1±458.8	3834.5±642.7	2509.9±2651.1	2530±314.7
Rate of Relaxation (cmH_2O/msec)	-1302±381.8	-2828.4±417.3	-2376.4±418.0	-2082±402.7
Heart Rate (beat/min)	184.6±29.5	228.3±85.4	191.1±48.9	196.1±14
After Treatment and Pre-ischemia				
LVDP (cmH_2O)	99.6±6	93.8±15.5	90.5±6.3	87.1±15.4
Rate of Contraction (cmH_2O/msec)	3051.9±916.7	2241±85.9	2065.5±351.3	1893.6±270.9
Rate of Relaxation (cmH_2O/msec)	-1750.7±449.4	-1779.6±161.3	-1365±379.7	-1510.8±246.4
Heart Rate (beat/min)	208.0±35.9	230.1±74.2	163.5±61.7	217.1±19.7
Post-ischemia				
LVDP (cmH_2O)	27.1±3.7	22.5±3.4	31.9±5.8	20.3±6.8
Rate of Contraction (cmH_2O/msec)	966.2±355.9	747.1±185.5	893±204.5	699.1±288.1
Rate of Relaxation (cmH_2O/msec)	-815.4±302.3	-626.6±172	-738.1±158.3	-578.4±240.6
Heart Rate (beat/min)	296.7±47.4	355.6±22.3	248.3±58.4	180.3±26.7

Table 4.2 The impact of different EDP concentrations on cardiac function

Chapter 5

General Discussion

5.1 Discussion

Many researchers have investigated the association between moderate consumption of fish oil and lower risks of CVDs (Lee et al., 2008; Lavie et al., 2009; Le Guennec et al., 2010). However, other studies raised concerns about the undesirable effects of n-3 PUFAs on human health (Riediger et al., 2009; Serini et al., 2011). The aim of this thesis is to investigate the protective role of acute administration of n-PUFAs on cardiac cells subjected to IR injury. As well, studies were performed to investigate the impact of DHA administration on mitochondria. In the present work, we failed to observe the protective role of n-3 PUFAs on cardiac functions of ex vivo hearts. Moreover, treatment caused a significant change in mitochondrial structure, as well as induction of apoptosis.

This thesis investigated the effects of DHA in response to IR injury in isolated hearts and cell culture models. We determined the potential mechanisms underlying DHA-mediated toxicity in H9c2 cells. Our results show that increasing DHA concentrations lead to significant decreases in cell viability. Increased DHA concentrations (0, 50 or 100 μM) are more toxic after anoxia-reoxygenation. DHA also activated caspase-8 and -3, suggesting that an apoptotic pathway caused cell death. More specifically, DHA-mediated cell injury targeted the mitochondria, causing a loss in mitochondrial membrane potential and increased fragmentation. We further investigated the cardioprotective effects of acute administration of DHA on isolated perfused hearts. DHA failed to protect hearts from IR injury, and high concentrations of DHA had significant effects on baseline cardiac functions (pre-ischemic), causing significant changes in LVDP, heart rate, rate of

contraction and rate of relaxation. Taken together, these data suggest that acute administration of high concentrations of DHA has an adverse effect on cardiac function and leads to apoptotic cellular death.

Many previous studies have reported the detrimental effects of different DHA concentrations in a variety of cell models. One of these studies done by Sandal et al (2009) and investigated the impact of acute administration of DHA on isolated cell viability of mouse thymocytes, showing that compared with other n-3 and n-6 fatty acids, 1 µM DHA has a strong and rapid (within 5 minutes) effect on reducing cell viability. The researchers thus associated cell death with increases in intracellular calcium $(Ca^{2+})_i$ concentrations. These results agree with our finding that DHA decreases cell viability in H9c2 cells. However, we did not find a reduction in cell viability in 1 µM DHA and normoxic conditions. We found that at the same DHA concentration, reductions in cell viability occurred at 24 h anoxia/16 h reoxygenation. In another study done by Kim et al (2005) who tested the effect of DHA (concentrations of 10 and 40 µM) on human umbilical vein endothelial cells (HUVECs). They found that DHA causes a significant reduction in cell viability and induces apoptosis by assaying TUNEL-positive cells. A similar cell line, ECV-304, which originates from HUVECs, has been used to examine the role of fatty acids in endothelium dysfunction (Masi et al., 2011). The researchers found that n-3 PUFAs reduce cell viability, agreeing with the findings of Kim et al (2005), as well as our results. However, Masi et al (2011) showed no significant effect of n-3 PUFAs on ROS production. Regardless of the cell type used by Kim et al (2005) and Masi et al (2011), DHA caused a negative

impact on some types of cells. Similarly, we showed significant cell death in H9c2 cells.

Mitochondria help maintain the proper function of tissues by ATP generation and contribute to cell death and survival (Nishida et al., 2010). Many studies have shown that n-3 PUFAs have a positive impact on mitochondrial function and structure (Pepe, 2005; Duda et al., 2009; Stanley et al., 2012). However, other studies, including ours, show that the opposite is true. (Khairallah et al., 2012) examined mPTP in rats fed DHA+AA for 10 weeks and found no changes in cardiac function. However, increasing the phospholipid content of the mitochondrial membrane with DHA+AA was detrimental to the animals and caused a significant increase in Ca^{+2} concentrations and mPTP opening (Khairallah et al., 2012). Another study done by Kim et al (2005) showed that mitochondria contribute to apoptosis by reducing $\Delta\Psi m$ in a time-dependent manner (4, 12 or 24 h) under high concentrations (5.5 g/day) of DHA; a significant increase in ROS production was also observed. Another study treated thymocytes with 20 μM PUFA and showed that cell death is caused by an increase in $[Ca^{2+}]_i$ released from the ER and ROS release from the mitochondria (Prasad et al., 2010). Our failure to prove the beneficial role of DHA is similar to the studies of Khairallah et al (2012) and Kim et al (2005) and could explain the enrolment of mPTP opening and $\Delta\Psi m$ depolarization and generation of ROS in cell death. In our study, time-dependent and dose-dependent reductions in $\Delta\Psi m$ were observed with DHA treatment.

Optic atrophy 1 (Opa 1), a key protein located in the inner mitochondrial membrane, helps to maintain the mitochondrial cristae structure. Evidence shows that mitochondrial fragmentation or failure of mitochondrial fusion leads to down-regulation of Opa 1 expression and release of cytochrome c as indicators of apoptosis (Chen and Knowlton, 2011). In the present study, we showed that mitochondrial fragmentation and the loss of mitochondrial $\Delta\Psi m$ occur in a time-dependent manner (Figures 4.8 and 4.10). We also demonstrated a significant reduction in Opa 1 expression by Western blot (Figure 4.14) after 24 h of treatment with DHA.

Interruption of mitochondrial fusion and fission proteins is involved in cardiac cell death, as shown by (Chen et al., 2009). H9c2 shows many similarities to rat primary cardiomyocytes, including electrophysiological properties, membrane morphology and protein expression (Watkins et al., 2011). Several techniques have been used to immortalize embryonic heart cells by transfection with the T antigen gene of an oncogenic virus (Allen et al., 2005). H9c2 cells have been used in many mechanistic studies to investigate the effect of toxic compounds on apoptotic activation (Watkins et al., 2011). (Chen et al., 2009) subjected H9c2 cells to 10 h hypoxia to assess Opa 1 expression and cytochrome c release by Western blot, results showed the relationship between hypoxia and reduction in Opa 1 expression and increases in cytochrome c release. These results are consistent with our findings in Figures 4.12 and 4.14. We were able to induce apoptosis using H9c2 cells with a toxic concentration of DHA. A similar cell line was used by Shin et al (2009) to investigate the role of leptin, an adipose-

derived hormone that is involved in energy homeostasis by preventing apoptosis induced by hypoxia/reoxygenation injury in cardiomyocytes. Leptin reduced changes in $\Delta\Psi m$ and prevented the release of cytochrome c, suggesting its protective role in preventing apoptosis. However, in our study, DHA failed to prevent damage resulting from IR injury.

Besides mitochondrial fragmentation, plasma membrane blebbing and loss of integrity are key morphological changes that occur in apoptotic cells (Kroemer et al., 2009; Whelan et al., 2010). H9c2 cells were used by Noritake et al (2012), who found that the toxic effect of alcohols causes plasma membrane blebbing and activation of apoptosis through mitochondria and caspase-3 activation. (Zhang et al., 2009) also showed how mitochondria are damaged after H9c2 cells are treated with doxorubicin (Dox), an anti-cancer agent. In a study done by Sardao et al (2009), apoptosis was induced by treating H9c2 cells with Dox to cause cardiomyopathy; mitochondrial fragmentation and caspase-3 activation were also observed. Using epifluorescence microscopy, Sardao et al (2009) also observed cellular rounding up and blebbing of the plasma membrane, both of which characterize apoptosis. In our work, we observed characteristic changes in the morphology of H9c2 cells after DHA treatment. Toxic concentrations of DHA caused cellular rounding up and shrinkage (Figure 4.4), indicating apoptotic cell death through caspase activation. As well, we detected cytochrome c release in the cytosolic fraction after cells were treated with DHA in the absence of a stressor, as shown in Figure 4.12. However, no significant changes in caspase-3

activity were observed after treatment of isolated hearts with DHA, indicating that inhibition in LVDP has a role different than apoptosis cell death.

Many experimental studies have shown the beneficial effect of chronic treatment of n-3 PUFAs on cardiovascular function (McLennan et al., 2007; Lavie et al., 2009; Russo, 2009). In our study, we investigated how DHA affects cardiac function. Our data demonstrated that high concentrations (50 and 100 μM) of DHA cause significant decreases in the heart rate, consistent with findings in pigs that were infused with DHA (45 mg total) and had their left anterior descending arteries subjected to 45 min occlusions and 180 min reperfusion (Xiao et al., 2008). Xiao et al. (2008) further found reductions in heart rate before and during coronary occlusion in the presence of DHA. Because DHA is highly lipophilic, it can defuse rapidly into the myocardium, leading to elevated intracellular Ca^{2+} and activation of the Na^{+}/Ca^{2+} exchanger that alters the contractility of cardiomyocytes (Xiao et al., 2008). In our study, we did not measure intracellular Ca^{2+}. Changes in cardiomyocyte contractility found by Xiao et al. (2008) could explain the significant changes we observed in hearts treated with high DHA concentrations and prior to IR injury. The ability of DHA to diffuse through the heart and affect its functions without changing caspase activity confirms its effects on the cell membrane and ion channels, which impact the contractility of the heart but not activation of caspases.

Any alteration in cell membrane composition will impact the cell physiological function significantly (Kang, 2007; Gorjao et al., 2009). (Sergiel et al., 1998) assessed the impact of a 6-week DHA diet on IR injury in rats,

demonstrating that heart membrane phospholipids are enriched with DHA. When the phospholipid membrane is enriched with DHA, depletion of n-6 PUFAs, more specifically AA, occurs, leading to decreased heart rates and post-ischemic functional recovery. The researchers thus suggested that these effects are due to low levels of AA and the adverse effects of increased DHA peroxidation. Similarly, a deficiency in cardioprotection was observed in isolated hearts of female mice fed fish oil for 4 weeks and subjected to IR injury (Huggins et al., 2009). These results are consistent with our findings and suggest the detrimental effects of DHA toward post-ischemic functional recovery.

A study done by Verkerk et al (2006) concluded that the effects of a diet rich in fish oil may depend on the condition of the consumer; they believed that fish oil may work as an anti-arrhythmic in case of heart failure but could be ineffective in acute ischemia cases. In our work, at low concentrations of DHA, no significant changes were observed in pre-ischemic treated hearts. Similarly, cells treated with DHA concentrations of 1 μM to 10 μM did not show negative effects (cell death) in normoxic conditions. However, when ischemia was prolonged to 24 h anoxia/16 h reoxygenation, significant cell death was observed. In contrast, we found that low concentrations of DHA could be similar to the amount normally consumed as part of the daily diet and have no effect under normal conditions; under ischemic conditions, however, such concentrations could be detrimental.

Cellular homeostasis depends on the membrane bilayer lipid structure and transmembrane protein function. Any alteration (increase or decrease) in the

membrane structure may have major and rapid effects, resulting in cell death (Sandal et al., 2009). Previous studies have reported the ability of DHA to embed itself in the cell membrane and impact intracellular activity, such as those of enzymes and ion channels (Kang, 2007). Scientists have reported that n-3 PUFAs can alter the permeability of a cell membrane by significantly affecting the cellular enzymes and ion channel functions (Gorjao et al., 2009). A study done by Owen et al (2004) reported that rats fed fish oil (1.25%, 2.25%, 2.50%, 5% and 10%) show marked dose-dependent increases in DHA in the phospholipid membrane and showed that diets with low contents of fish oil (1.25% of the total diet) could have significant impacts on the myocardial phospholipid composition over a very short period of time (2 days). In our work, we showed that high concentrations of DHA could affect cardiac function very quickly (less that 20 minutes) after acute treatment, indicating the rapid ability of DHA to change the membrane composition, which impacts cell enzymes and ion channels.

Increased lipid peroxidation is associated with alterations to membrane proteins, receptors and transporters. Lipid peroxidation also causes formation of ROS and loss of cell integrity (Yang et al., 2003; Uchida, 2007). For example, increased lipid peroxidation changes the tocopherol content and Mg-K-ATPase activity, as observed by Calviello et al (1997). They also reported that retroconversion of n-3 PUFAs to n-6 PUFAs is limited to the heart, proposing that DHA may be acylated into heart phospholipids but not retroconverted into n-6 PUFAs. The researchers further suggested that treatment with EPA and DHA must be done at low doses to avoid massive changes in membrane fatty acids that

could cause them to become more susceptible to oxidation. These findings may explain why high doses of DHA cause massive changes in perfused hearts before ischemia and marked cell death under normoxic conditions. Other studies have not found that PUFAs cause damage as a result of lipid peroxidation. For example Ando et al (2000) fed rats with fish oil for 6 weeks and collected their organs, including the heart, afterward. No significant production of lipid peroxidase was found. Another study done by Calzada et al (2010) found an increase in 4-hydroxy-nonenal concentrations in healthy men advised to take 1600 mg DHA/day for 2 weeks. (Calzada et al., 2010) thus recommended a DHA intake of 800 mg/day or lower to avoid peroxidation. Although many studies have reported the negative effects of lipid peroxidation, the role that PUFAs play in this process requires further study before their relationship can be fully understood.

Dual damage could occur in cardiomyocytes as a result of free radical formation from hypoxia/reoxygenation and the susceptibility of PUFAs to peroxidation (O'Farrell and Jackson, 1997; Bordoni et al., 2005). Rats under a PUFA diet for 6 weeks (10% of the total weight) were used by O'Farrell and Jackson (1997) in an experiment during which hearts were isolated and subjected to IR injury. O'Farrell and Jackson used an assay of thiobarbituric acid-reactive substances (TBARS) to measure the production of lipid peroxidation and found that animals fed n-3 PUFAs have more positive TBARS than do other PUFAs. Another study by Bordoni et al (2005) related the enhancement of peroxidation in the presence of PUFAs to hypoxia using primary heart cells subjected to hypoxia. During reoxygenation, PUFAs were administered to the hearts. Although we did

not measure levels of lipid peroxidation in our work, these previous findings agree with and explain our data. We incubated cells with 1 µM to 10 µM DHA in normoxic conditions and found no differences in cell viability. However, when the cells were subjected to 24 h anoxia/16 h reoxygenation under the same DHA doses, significant cell death occurred, a finding that could be related to the presence of stressors and susceptibility of DHA to peroxidation.

Judé et al (2003) demonstrated that the pharmacological properties of n-3 PUFAs are very sensitive to oxidative compounds. In their work, DHA (10 µM) was acutely applied to an isolated rate ventricular myocyte with or without compounds that prevent peroxidation. The researchers found that DHA peroxidation products could alter the fluidity of the membrane by blocking the fast sodium current and modifying channel activity. As well, DHA caused a blockade of transient outward current (I_{TO}) and the effect on K^+ channels was more likely because of DHA peroxidation and not due to the DHA itself. To relate this finding to the present work, changes in the ion channels as a result of DHA could impact heart rate, systolic and diastolic force, and subsequently LVDP.

Induction of cell death by DHA-induced lipid peroxidation could explain the potential mechanism behind the detrimental effects that we found in the current work. The detrimental effects of DHA (in terms of heart function or cell viability) may be avoided by an antioxidant compound, such as α-tocopherol (vitamin E), to reduce the occurrence of peroxidation (Valk and Hornstra, 2000). Ingested PUFAs are less likely to cause peroxidation, as the circulatory system has high levels of antioxidants (O'Farrell and Jackson, 1997; Ando et al., 2000;

Jude et al., 2003). However, Ottestad et al (2011) found that 3 to 7 weeks of treatment with oxidized n-3 fish oil do not significantly change markers of oxidative stress, lipid peroxidation and inflammation that influence health risks (including CHD). In the present work, we did not measure ROS or lipid peroxidation generation, but Ottestad's findings do not match some studies that found a detrimental effect of fish oil because of ROS and lipid peroxidation generation.

Some studies have found no relationship between limiting adverse cardiovascular events and increasing levels of n-3 PUFAs. Interest in understanding the effect of n-3 PUFAs in CVD and their connection to neurological damage has suggested that there is an increased risk of myocardial infraction caused by long-term supplementation with fish oil. Such studies hypothesize that this finding could be due to the presence of toxic compounds, such as fat-soluble methylmercury (Hooper et al., 2006). A group of researchers Burr et al (2003) studied the role of fish oil in reducing the risk of cardiac death and recommended that patients with angina take two 3 g portions of fish or fish oil capsules, daily, for 6 months. These researchers concluded (with no explanation) that patients who consume large amounts of fish oil are at a higher risk of cardiac death. The lack of beneficial effects of n-3 PUFAs was also shown by Brouwer et al (2009), who advised patients with implantable cardioverter-defibrillators to take 2 g of fish oil a day for 6 months. More clinical trials need to be done to explain why taking n-3 PUFAs has few benefits.

The DHA concentration used in the present work was similar to the concentration used in previous studies, such as those of (Kim et al., 2005; Masi et al., 2011). It is estimated that endogenous n-3 PUFAs levels are lower than 10 μM. Following daily supplementation of 0.4 g of n-3 PUFAs, the expectation is that 50% levels would be reached in the tissue phospholipid, as shown in (Yates et al., 2011). The present work showed that DHA concentrations 10 μM and lower could be similar to physiological doses and may be safe in the present or absence of ischemia/anoxia. Data generated from the current work demonstrate that concentrations of DHA $\geq$ 50 μM cause undesirable effects.

CYP plays an essential role in generating the EDP of DHA (VanRollins et al., 1989; Halestrap, 2009). EDP is known to possess numerous properties that impact cardiovascular function. For example, evidence has shown that EDP plays a significant role in inhibiting platelet aggregation and TXB_2 formation at doses of 0.7 μM to 1.5 μM in human platelet incubations and platelet aggregation assays (VanRollins, 1995). In addition, EDP has been demonstrated to possess anti-arrhythmic properties in a model of neonatal rat cardiomyocytes (Arnold et al., 2010b). EDP can act as a potent dilator of rat coronary arteries, which may be important for hypertensive conditions (Ye et al., 2002). However, the cellular mechanism(s) of action of EDP remain largely unknown. We hypothesize that EDP has cardioprotective properties, such as the ability to inhibit platelet aggregation and act as a vasodilator, and may play a protective role after IR injury. In our study, we sought to determine whether or not the adverse effects of DHA could be attributed to EDP. Perfusion of hearts with EDP did not affect their

pre-ischemic or post-ischemic functions. Similarly, no significant changes in the cell viability of H9c2 cells were observed with different doses of EDP and subjected to 24 h anoxia/16 h reoxygenation. In our model, EDP did not cause detrimental or beneficial effects. As such, we cannot confirm that EDP plays a role in preventing damage from IR injury.

In summary, our data indicate that acute administration of high DHA concentrations is detrimental to isolated hearts and H9c2 cultured cells in the presence or absence of IR injury. We found that high DHA concentrations are toxic and induce apoptosis by causing obvious changes to the mitochondria, resulting in cellular dysfunction. A mechanistic study of the cause of cell death and the long-term effects of n-3 PUFAs on cardiomyocytes should be evaluated in future studies. We highly recommend that monitoring of levels of oxidized PUFAs be done in people who consume large amounts of fish oil to avoid the undesirable side effect of PUFAs.

5.2 Limitations

Similar to any study, the present work has some limitations that must be addressed. One of the major limitations we found is the difference in the reactions of H9c2 cells and isolated heart tissues to DHA treatment. For example, significant activation of caspase-3 was observed in H9c2 cells treated with DHA but no such activity was found in heart tissue treated in the same manner. This difference in response could be due to differences in the biological and physiological properties of H9c2 cells and heart tissues. H9ce cells are immortalized cells that behave more like cancer cells. Preliminary work done in

our laboratory, to confirm differences in the reactions of H9c2 cells and primary cells to DHA treatment, indicates that DHA does not induce cell death in primary neonatal cardiomyocytes.

As shown in many studies, a major contributing factor describing the detrimental effect of n-3 PUFAs suggests the generation of lipid peroxidation is important. In these studies, formation of lipid peroxidation was detected by TBARS experiments. In the present study, we observed the harmful effects of DHA on H9c2 cells but we could not confirm that these effects were due to lipid peroxidation.

We were able to show the dose-dependent effects of increasing concentrations (1, 5, 10, 50 and 100 μM) of DHA in the cell viability of H9c2 cells. However, one of the limitation of the present work is not assessing the effects of the concentrations between 10 μM and 50 μM DHA on cell viability in H9c2 cells. We might find gradual decreases in cell viability if cells were treated with 30 μM and 40 μM DHA. Regarding the perfused heart, we found slight increases in the functional recovery of heart treated with 10 μM DHA. However, we cannot predict the level of functional recovery of hearts treated with DHA concentrations lower than 10.

5.3 Future studies

In our study, we found that acute administration of high concentrations of DHA has detrimental effects, causing losses in mitochondrial integrity and function in H9c2 cells. High concentrations of DHA also caused significant changes in heart functions. To continue the present study, more experiments need

to be done to provide an explanation for the cell death observed. We must confirm whether or not H9c2 cell death is due to lipid peroxidation. Another interesting point that requires attention in the present work is the determination of markers of change in heart tissues treated with high concentration of DHA. For example, we can assess the elevation of intracellular Ca^{+2} levels that lead to changes in different heart function.

Many studies (Das and Puskas, 2009; Le Guennec et al., 2010; Shukla et al., 2010) have proven that fish oil plays a role in cardioprotection. Our work could be further expanded by testing the impact of feeding mice with different concentrations of n-3 PUFAs, specifically EPA and DHA. After 6 to 8 weeks of feeding, the mouse hearts may be isolated and perfused and tested for heart functions after subjecting to IR injury. We need to determine whether or not feeding exerts protective effects. For example, which n-3 PUFA doses have better or poorer effects on heart function? What is the impact of feeding on mitochondrial function?

Some limitations have been reported from feeding studies. When feeding animals different diets, variations may be found between the experimental groups because it is difficult ensure that the same amount of food is delivered to the different experimental groups. As a result, the properties and the amount of food consumed will be not accurate (Das and Puskas, 2009; Menesi et al., 2009). To address this issue, a new animal model was established to enhance or increase n-3 PUFAs in the animal tissues and cells. This model is called the *fat-1* transgenic mouse. The *fat-1* mouse has the ability to convert n-6 PUFAs to n-3 PUFAs by

addition of double bonds to the hydrocarbon chains of n-6 PUFAs. The *fat-1* mouse can maintain a normal diet. In other words, this animal does not need to an n-3 PUFA diet (Kang, 2007). It will be interesting to investigate whether or not the *fat-1* mouse has a protective role after IR injury. As well, we are interested in comparing the recovery of *fat-1* mouse, as well as the mechanism of such recovery, in a feeding study with acute treatment with n-3 PUFAs.

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