Combination treatment with conjugated linoleic acid and nitrite protects against myocardial infarction.

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COMBINATION TREATMENT WITH CONJUGATED LINOLEIC ACID AND NITRITE PROTECTS AGAINST MYOCARDIAL INFARCTION

By

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M.D. , Tbilisi State Medical University

A Dissertation
Submitted to the Faculty of the
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for the Degree of

Doctor of Philosophy in Physiology and Biophysics

Department of Physiology & Biophysics

University of Louisville

Louisville, KY

August 2015
DEDICATION

This dissertation is dedicated to

my parents

Neli Metreveli and Ushangi Qipshidze

And

my husband

David A. Kelm

for believing in me and encouraging me throughout my life.
ACKNOWLEDGEMENTS

The work within this dissertation represents the culmination of years of training, during which time I have been extremely fortunate to receive help from my multi-talented and supportive mentor, Dr. Marsha Cole. Thanks to Dr. Cole for providing me with an outstanding pre-doctoral training, intellectual challenges, her well-timed humor, and endless support of my current and future endeavors. I would also like to thank my committee members: Dr. Schuschke, Dr. Bhatnagar, Dr. Keller, Dr. Maldonado, and Dr. Boyd for their support and sage advice. I am especially grateful to Dr. Keller and Dr. Bhatnagar for their guidance and support. The Department of Physiology has provided the support and equipment I have needed to produce and complete my thesis. Finally, I would like to thank my parents, sister and husband for their encouragement.

To acknowledge the team approach to science, the following text frequently uses the term “we” to describe collaborative experiments executed during my doctoral thesis work.
ABSTRACT

COMBINATION TREATMENT WITH CONJUGATED LINOLEIC ACID AND NITRITE PROTECTS AGAINST MYOCARDIAL INfarction

Natia Qipshidze Kelm
August 5, 2015

According to the CDC, the most common type of heart disease is coronary artery disease, which frequently leads to myocardial infarction (MI). Therapeutic approaches to lessen the resulting cardiovascular injury associated with MI are limited. Recently, the management paradigm for cardiac injury has entered the molecular era and microRNAs (miRNAs) have been shown to act as negative regulators of gene expression by inhibiting mRNA translation and/or stimulating mRNA degradation. A single miRNA can modulate physiological or disease phenotypes by regulating whole functional systems. Importantly, miRNAs can regulate cardiac function, thereby modulating heart muscle contraction, heart growth and morphogenesis. MicroRNA-499 (miRNA-499) is a cardiac-specific miRNA that when elevated causes cardiomyocyte hypertrophy, in turn preventing cardiac dysfunction during MI. Previous studies revealed that the combination treatment of conjugated linoleic acid (cLA) and nitrite preserved cardiovascular
function in mice. Therefore, we hypothesized that cLA and nitrite may regulate miRNA-499, thus providing cardiac protection during MI. To test this hypothesis, 12-week old mice were treated with cLA (10 mg/kg/d-via osmotic mini-pump) or cLA and nitrite (50 ppm-drinking water) 3 days prior to MI (ligation of the left anterior descending artery). Echocardiography and pressure-volume (PV)-loop analysis revealed that cLA and nitrite-treated MI mice had improved heart function (10 days following MI) compared to untreated MI mice. Treatment with cLA and nitrite significantly induced levels of miRNA-499 compared to untreated MI mice. In addition, treatment with cLA and nitrite abolished MI-induced protein expression of p53 and dynamin-related protein-1 (DRP-1). Moreover, the antioxidant enzyme expression of heme oxygenase-1 (HO-1) was elevated in MI mice treated with cLA and nitrite compared to untreated MI mice. Confocal imaging on heart tissue confirmed expression the levels of HO-1 and p53. Taken together, these results suggest that therapeutic treatment with cLA and nitrite may provide significant protection during MI through regulation of both cardiac specific miRNA-499 and upregulation of phase 2 antioxidant enzyme expression.

As we demonstrate in our study cLA and nitrite co administration decreased apoptosis though the HO-1 and/or miRNA-499 pathway. To investigate more deeply the role of HO-1 and/or miRNA-499 in apoptosis, we used HO-1 Tg and HO-1 KO mice. Our data supported the hypothesis that HO-1 regulates miRNA-499 levels and thus decreases apoptosis after MI. As others and we have demonstrated before, MI is known to cause cardiomyocyte ischemia, in turn, leading to cardiomyocyte apoptosis [1, 2]. The current study
extends previous findings by demonstrating that ischemia causes an increased ratio of Bax/Bcl-2 following MI in non-treated C57 mice and in HO-1 KO mice. Our data demonstrate that in HO-1 KO mice the expression of miRNA-499 is not detectable.

Overall, these data reveal links among p53, HO-1, miRNA-499, and Drp1 with regard to regulation of the apoptotic programmed cell death in the heart. Taken together, these results suggest that therapeutic treatment with cLA and nitrite may provide cardiac protection during MI through the regulation of induction of cardiac specific HO-1 expression, which further regulated cardiac specific miRNA-499.
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A. Myocardial infarction

Myocardial infarction (MI) caused by coronary artery blockage is the major cause of death worldwide [3]. Over the last two decades, health care professionals, consumers, and payer organizations have sought to improve outcomes for patients hospitalized with acute MI [4]. In the United States (US) alone, nearly 8 million people have a history of MI [5]. Subsequently, in 1990 the American College of Cardiology and the American Heart Association jointly published the first in a series of clinical practice guidelines for MI that include a significant emphasis on evidence-based hospital care [6].

In 1992 the Health Care Financing Administration, now the Centers for Medicare & Medicaid Services (CMS), initiated an ongoing national effort, aided by quality improvement organizations, to measure and improve hospital care for patients with acute MI [7]. According to the World Health Organization, ischemic heart disease is one of the leading causes of death worldwide, with about 3.8 million men and 3.4 million women dying yearly from this disease. In the US
ischemic heart disease resulting from coronary artery disease is devastating, with 1.5 million US citizens developing MI-s that account for nearly 200,000 deaths per year [8, 9]. The National Heart, Lung, and Blood Institute estimate that an average of 14.2 years of life may be lost due to heart attack [10].

Acute MI remains a leading cause of morbidity and mortality worldwide [11]. MI occurs when myocardial ischemia, a diminished blood supply to the heart, exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis [12]. Ischemia at this critical threshold level for an extended period results in irreversible myocardial cell damage or death [13]. Critical myocardial ischemia can occur as a result of increased myocardial metabolic demand, decreased delivery of oxygen and nutrients to the myocardium via the coronary circulation, or both [14]. Acute MI results from blockage of a coronary artery leading to insufficient blood supply to cardiac tissue, ultimately resulting in cardiomyocyte death [15] and cardiac dysfunction [13]. MI is characterized by significant changes in gene expression, many of which represent adaptive or maladaptive responses to stress [16-20]. The resulting cardiac stress induces rapid changes in gene expression immediately following MI [21]. Cardiomyocyte cell death is a consequence of myocardial injury, which occurs as early as the initiation of acute MI [22]. Cardiomyocyte death or apoptosis is a key factor in transition from cardiac hypertrophy to heart failure [23].
B. Apoptosis and myocardial infarction

Apoptosis or programmed cell death is a highly regulated and energy requiring process [24]. Apoptosis is characterized by shrinkage of the cell and the nucleus [24]. The nuclear chromatin is cut into sharply defined masses, and eventually breaks up into the small particles [25]. At this stage, extensions bud out from their membranes, which eventually seal off to form membrane enclosed vesicles, called apoptotic bodies, containing condensed cellular organelles and nuclear fragments [25, 26]. These apoptotic bodies are either rapidly phagocytosed by neighboring cells or undergo degradation, which resembles necrosis in a process called secondary necrosis [26]. However, apoptosis is generally considered not to trigger an inflammatory response [27].

Apoptosis plays a role in the tissue damage after MI, it has pathological and therapeutic implications [28]. Apoptosis is highly regulated and the consequences of the cellular mechanisms that control apoptosis during and/or after MI are well understood [26, 29]. Thus, the apoptotic pathway becomes an important therapeutic target with regard to attenuation of tissue damage in patients with MI and consequent heart failure.

During MI-induced heart damage, cardiomyocyte apoptosis contributes to dilatation of the infarcted chamber, and thus leads to cardiac dysfunction or eventual heart rupture following MI [30]. MI represents one of the major etiologies that emphasizes the expansion of congestive heart failure [31]. Damage of cardiomyocytes secondary to prolonged ischemia has been thought to be
resulted from the manifestation of necrosis [32]. Whereas this form of cell death remains a main cause of tissue injury, recent studies have suggested that damage of cardiomyocytes after MI can also be caused by apoptosis [33-37]. Overall, myocyte death in MI is attributed to necrosis, but recently myocardial apoptosis has been observed in humans during acute MI [36, 37]. Apoptosis is the early manifestation of cell death in infarcted myocardium [38], which is primarily seen in cardiomyocytes in hypoxic regions during MI [36]. p53 is a well-known transcription factor which mediates apoptosis by initiating the expression of pro-apoptotic genes, including Bax, caspase, etc. [39]. Previous studies have demonstrated that p53 transcriptional activity is enhanced in MI [40] and that p53 plays a critical role in the regulation of hypoxia-induced apoptosis of cardiomyocytes [1]. Importantly, p53 signaling is proposed to be part of the pro-apoptotic pathways which eventually lead to cardiac apoptosis [39]. Enlargement of the LV chamber is a distinctive alteration of the failing heart regardless of cause [31]. For example, chronic ventricular dilatation and failure can be an outcome of long-standing volume overload. LV chamber dilation is associated with the loss of cardiac myocytes [41]. Previous studies have shown that myocardial stretch is associated with cardiomyocyte apoptosis [42, 43]. More recent studies have shown that myocardial stretch alone, under conditions of acute or chronic volume overload, may be associated with cardiomyocyte loss through apoptosis [31].

Bcl-2, Bax and p53 have been recognized to play a significant role in the management of cell death and apoptosis [44]. Apoptosis is a tightly regulated
process, as previously mentioned, and the imbalance between Bax and Bcl-2 proteins decides the outcome of the cell [38]. The expression of these regulatory proteins, Bcl-2 and Bax, has been studied following MI in cardiac tissue [45]. Bax is a member of the Bcl-2 family and its overexpression accelerates apoptosis [15, 46]. It also counters the death repressor activity of Bcl-2, the Bcl-2 family proteins are involved in the p53 apoptotic pathway and the balance between those positively and negatively regulatory proteins is essential for forming apoptosis [47]. Bcl-2 is known as anti-apoptotic protein, and Bax is widely described as a pro-apoptotic factor, thus the balance between Bax and Bcl-2 contributes to the signaling of programmed cell death through apoptosis [47, 48]. It has been shown that Bcl-2 expression can inhibit apoptosis in ischemic cardiomyocytes after MI [49, 50] and myocardial ischemia induces a reduction in the expression of Bcl-2 protein and increases in the expression of Bax and p53 proteins [51, 52]. Therefore an imbalance in anti- and pro-apoptotic proteins expressed during myocardial ischemia is involved in initiating myocyte cell death [53, 54]. Overall, if the Bax to Bcl-2 ratio is high, this may indicate apoptotic cell death infarcted myocardium [45].
C. miRNA-499 protects the heart from apoptosis

MicroRNAs (miRNAs) quantitatively regulate mature-RNAs, which affect the cardiac transcriptional output and cardiac function [55]. miRNAs are endogenous, single-stranded non-coding RNAs ranging 18 to 24 nucleotides in length, that play an important role in gene regulation [55, 56]. In screening for miRNAs enriched in the heart, miRNA-499 is abundant and is known to be an evolutionarily conserved muscle-specific microRNA that is encoded within the intron of myosin heavy chain and is highly expressed in the cardiac ventricles [55-57]. Plasma miRNA-499 is a known biomarker of acute MI, as plasma miRNA-499 has been observed in individuals with MI [58-60].

The balance between cell proliferation and apoptosis is required for living cells and miRNA-499 plays a big role in maintaining this balance [61]. Previous studies show that overexpression of miRNA-499 reversed enhanced proliferation and shows an anti-apoptotic effect in cardiomyocytes [61].

Ultimately, heart function is highly dependent on ATP generation. The heart is enriched with mitochondria that provide the energy required for cardiomyocyte function [62]. Therefore, it is known that mitochondria play a critical role in development and progression of many cardiac diseases such as hypertrophy and MI. Previous studies suggest that mitochondria are highly dynamic and that changes in mitochondrial shape can affect a variety of biological processes such as apoptosis and fibrosis [63, 64]. Mitochondria are organelles which constantly undergo fission and fusion [63-65]. These two
opposing processes are regulated by the mitochondrial fusion proteins mitofusin and the mitochondrial fission protein Drp-1 [62]. During apoptosis, Drp-1 foci accumulates on mitochondria and can enhance mitochondrial fission [66, 67].

miRNA-499 has been shown to be involved in inhibiting apoptosis and MI induced by anoxia and ischemia through mechanisms involving p53 and Drp-1 in accomplishing apoptosis in the MI heart [57]. The modulation of miRNA-499 may be a novel therapeutic approach to treat apoptosis-related cardiac disease, including MI [68, 69].

D. Cardio-protective effects of fatty acids

Conjugated linoleic acid (cLA) refers to a group of positional and geometrical isomers of octadecadienoic acid, with two alternating double bonds. Rumen bacteria has the unique ability to convert linoleic acid into cLA via an enzymatic isomerase reaction, and therefore at least 28 possible isomers of linoleic acid as a naturally occurring trans fat are possible, and it is commonly found in ruminant animal sources such as beef, lamb, and dairy products (e.g., milk and cheese) [70]. Incidentally this is also where the concentrations are highest -beef, lamb, and dairy products [71-73]. Seafood, pork, most poultry products and vegetable oils are not notable sources of cLA [72, 73]. cLA has also
been identified as occurring naturally in foods such as white button mushrooms [74] and pomegranate seed oil [73].

In addition to ingestion through ruminant food products, cLA is commercially available as a dietary supplement, prominent isomers are cis-9, trans-11-cLA and trans-10, cis-12-cLA, but cis-9, trans-11 accounts for 72-94% of total cLA in foods [9, 72]. There is emerging evidence that individual cLA isomers may be responsible for specific biological or biochemical changes in the body [75].

Numerous studies have reported positive effects of cLA consumption, including weight loss in obese individuals [76], modulation of immune function [77, 78], anti-carcinogenic activity [79], protection against atherogenesis [80, 81], reversal of atherosclerosis [82], and normalization of glucose and insulin homeostasis in pre-diabetic animal models [83, 84]. However, recent studies from our lab suggest that cLA lowers survival in a murine model of myocardial ischemia, causing life threatening spontaneous ventricular tachycardia (VT) and sudden cardiac death (SCD) (Fig. 1). In addition, we have previously shown that cLA treatment lowers physiological nitric oxide (NO) levels and impairs heart function in aged mice [85].
E. Heme oxygenase-1 in cardiovascular disease

Recent literature suggests that dietary cLA and nitrite supplementation in rodents elevates nitrated-cLA (NO$_2$-cLA) levels in the plasma and tissues, inducing heme oxygenase-1 (HO-1) expression in the target tissue [86]. HO-1 catalyzes the oxidation of heme, producing biliverdin, iron, and carbon monoxide (CO) [87, 88]. Importantly, biliverdin is converted to bilirubin, a known potent antioxidant. Additionally, iron can be sequestered by ferritin, leading to off target anti-apoptotic effects and carbon monoxide has similar characteristics to nitric oxide, facilitating numerous biological functions including anti-inflammatory effects [88-90]. Induction of HO-1 is considered an important cardioprotective response in the failing heart that reverses pathological LV remodeling, and this effect is mediated through inhibition of apoptosis [90]. Increased expression and activity of HO-1 represents a therapeutic strategy for amelioration of LV failure [90]. It has been shown that induction of HO-1, eliminates inflammatory response and apoptotic events after MI [91]. HO-1 increases oxidative stress resistance and eliminates apoptosis [92]. Apoptosis consists of multiple mechanisms during heart failure that produces increased activity of extrinsic pathways of cell death [93]. HO-1 becomes a very important part of treatment to reduce apoptosis after MI.
CHAPTER II

HYPOTHESIS AND SPECIFIC AIM

**Overall aim**

MI affects over five million individuals in the US and remains a significant and yet unsolved health problem. MI results in an insufficient blood supply to cardiac tissue resulting in myocardial cell death and fibrosis [12, 94, 95]. MI is characterized by significant changes in gene expression, many of which represent adaptive or maladaptive responses to stress [16-20]. p53 is a well-known transcription factor which mediates apoptosis by activating the manifestation of pro-apoptotic genes [96]. Thus, p53 becomes an important therapeutic target, with regard to attenuation of cardiac apoptosis and consequent heart failure.

Recent literature suggests that dietary conjugated linoleic acid (cLA) and nitrite supplementation in rodents elevates nitrated cLA (NO$_2$-cLA) levels in the plasma and tissues, inducing heme-oxygenase-1 (HO-1) expression in the target tissue [86]. HO-1 has anti-apoptotic effects and facilitates numerous biological functions including anti-inflammatory effects [88-90]. In addition, miRNA-499, a
cardiac specific microRNA, can attenuate apoptosis via regulation of the p53 pathway [57]. Present data indicates that co-treatment with cLA and nitrite is cardio-protective through increased expression of miRNA-499 and HO-1, although the mechanism is unclear [97].
HYPOTHESIS

Co-treatment with cLA and nitrite is protective in heart failure through induction of HO-1 expression in cardiac tissue, leading to regulation of miRNA-499 expression and a reduction of myocyte apoptosis.

To address this hypothesis, this work will examine two aspects of the mechanism(s) involving HO-1 mediated miRNA-499 upregulation and heart function improvement following MI.

Two specific aims are proposed to test this hypothesis:

Specific aim 1: To determine whether the co-treatment with cLA and nitrite protects heart function in mice following MI.

Hypothesis: Co-treatment with cLA and nitrite reduces LV volume and diameter in mice following MI.

Specific aim 2: To determine whether co-treatment with cLA and nitrite increases HO-1 level in cardiac tissue, thereby increasing miRNA-499 and inhibiting p53-induced apoptosis.

Hypothesis: HO-1 expression increases miRNA-499 and provides a cardio-protective effect.
CHAPTER III

MATERIALS AND METHODS

A. Materials:

cLA (cis-9, trans-11 was purchased from NU-CHEKPREP, INC (Elysian, MN). Antibody against 4 Hydroxynonenal was purchased from Abcam (Burlingame, CA). Antibody against GAPDH was purchased from Trevigen, (Gaithersburg, MD), antibody against p53 was purchased from Calbiochem, (Billerica, MA) and antibody against HO-1 was purchased from Stressgen Biotechnologies, (Farmingdale, NY). TUNEL assay kit was purchased from Promega (Fitchburg, WI).

B. Animals:

Mice were fed standard chow and water ad libitum. All animal procedures were reviewed and approved by an independent Institutional Animal Care and Use Committee of the University of Louisville, School of Medicine. In addition, all studies were performed in accordance with animal care and use guidelines of the National Institutes of Health.
C. Mouse model of MI:

Male C57BL/6 mice, 10- to 12-wks old, were anesthetized with isoflurane, intubated and ventilated with CWE advanced ventilator (Webster,TX). Body temperature was maintained with an Indus Temperature feedback/surgical table and ECG system. Aseptic procedure was used for preparation of the surgical site through scrubbing with a 0.8% chlorhexidine solution. A left thoracotomy was performed via the fourth intercostal space and the lungs retracted to expose the heart. After opening the pericardium, the left anterior descending (LAD) coronary artery was ligated with an 8–0 silk suture near its origin between the pulmonary outflow tract and the edge of the atrium. Ligation was deemed successful when the anterior wall of the left ventricle (LV) became pale in color. The lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy side was closed in layers. The lungs were re-expanded, and the chest was closed. The animals were removed from the ventilator and allowed to recover on a heating pad. Mice were checked daily for signs of pain or distress and buprenorphine at 0.05mg/kg SQ is given before and every 12h for 48 hours. Mice were treated with cLA (10mg/kg/d-via osmotic mini-pump) or cLA + nitrite (50ppm-drinking water) 3 days prior to ligation of the LAD artery and treatment continued 10 days after ligation (Fig.2).
D. **Measurement of Cardiac function:**

1. **Echocardiography:**

   Mice were anesthetized with inhaled isoflurane and chest hair removed for baseline pre-MI and 10 days post-MI. Ultrasounds were obtained using a VisualSonics Vevo 2100 and a 40 MHz linear probe. Wall thickness and left ventricular cavity size were measured in at least 3 beats from each projection and averaged. Interventricular septum and posterior wall thickness, as well as internal dimensions at diastole and systole were measured. In addition, LV fractional shortening, relative wall thickness, and LV mass were calculated from the M-mode measurements [98].

2. **In vivo hemodynamic measurements:**

   Isoflurane was used to anesthetize the mouse and conductance readings were made for ~15-20 min prior to harvesting heart tissue. Briefly, the mouse was placed in a supine position on a 37°C pad under the surgical microscope and the limbs were restrained with tape. A 0.5cm skin incision was performed in the right neck area and the carotid artery was isolated using silk sutures. The cranial aspect of the carotid artery was ligated and a microsurgical clip was placed on the proximal carotid artery for hemostasis. An arteriotomy was performed with microsurgical scissors, and a 1.2 French conductance catheter
(Transonic, London, ON, Canada) was introduced into the carotid artery and advanced retrograde across the aortic valve into the LV. The catheter was advanced under continuous hemodynamic monitoring to issue proper placement in the LV. The catheter was secured within the carotid artery with the proximal suture. LV pressure-volume loops were recorded in the steady state. Loops were recorded using the iWorks data acquisition software package, and analyzed using the LabScribe2 pressure-volume data analysis software package (iWorks, St. Albans, Vermont).

E. Histology and confocal microscopy:

Hearts were collected from the mice and thoroughly washed in PBS. Using Peel-A-Way disposable plastic tissue embedding molds (Polysciense Inc, Washington, PA) filled with tissue freezing media (Triangle Biomedical Sciences, Durham, NC), hearts were preserved and stored at -80 C until analysis. Tissue sections (5 µm in thickness) were made using a Leica CM 1850 Cryocut microtome (Bannockburn, IL, USA). Sections were placed on super frost plus glass slides, air-dried, and processed for Immunohistochemistry (IHC) staining.
1. **Immunohistochemistry:**

   Immunohistochemistry and laser-scanning confocal microscopy were used to visualize MI-induced changes in p53 and HO-1 expression and localization. Immunohistochemistry was performed on frozen tissue sections using a standard IHC protocol. Primary antibodies were applied overnight (anti-p53, Calbiochem and anti HO-1, Stressgen). Secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Carlsbad, CA) were applied for protein immunodetection. Stained slides were analyzed for fluorescence using a laser scanning confocal microscope (Olympus FluoView-1000, objective 40X) set at the appropriate filter settings. The total fluorescence (green or red) intensity in 5 random fields (for each experimental sample) was measured with image analysis software (Image-Pro Plus, Media Cybernetics). Fluorescence intensity values for each experimental group were averaged and presented as fluorescent intensity units (FIU).

2. **TUNEL assay:**

   LV frozen sections were defrosted, and rehydrated with xylene and graded alcohol series. The sections were stained using the Apoptosis Detection System (Promega, Madison, WI) per the manufacturer’s instructions. Briefly, the sections were incubated with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP. To identify cardiomyocytes, sections were incubated with mouse
anti-myosin heavy chain monoclonal antibody (Chemicon International, Temecula, CA). Finally, to identify all nuclei (non-apoptotic and apoptotic), sections were stained with propidium iodide (Sigma-Aldrich, St Louis, MO). The samples were analyzed under a fluorescence microscope. Four sections randomly picked from each of four pieces were analyzed per animal. Cardiomyocyte nuclei were determined by random counting of 10 fields per section. The number of apoptotic nuclei was calculated per 10,000 cardiomyocytes.

F. Real-Time Quantitative PCR:

Total heart tissue was isolated with TRIzol and RNA was isolated. Complimentary DNA was isolated using oligo (dT) primers (Promega Corporation, Madison, WI) and stored at -80° C until experiments were performed.

Reactions were done using 2000ng/uL cDNA and SYBR Green Master Mix (BioRad Laboratories, Hercules, CA). BioRad CFX Manager (BioRad Laboraties, Hercules, CA) software was used to analyze results. Gene expression was normalized with mRNA expression of 18S. Samples were analyzed in triplicate using N=3 for each independent experiment.
Primers:

Mouse ANP:
- Forward: 5'-TGACAGGATTGGAGCCAGACAGAG-3'
- Reverse: 5'-AGCTGCGTGACACACCAAGAAG-3'

Mouse BNP:
- Forward: 5'-ATCGGATCCGTCAGTCGTTTG-3'
- Reverse: 5'-CCAGGCAGAGTCAGAAACTGGAG-3'

Mouse miRNA-499
- Forward: 5'-GGG-TGG-GCA-GCT-GTT-AAG-3'
- Reverse: 5'-AGG-CAG-CAG-CAC-AGA-CTT-G-3'

Mouse DRP-1
- Forward: 5'-AAA CTC CTA TCA CGC TCA TCA-3'
- Reverse: 5'-CTC ATC CTC CAC GCA TCC T-3'

Mouse Bax
- Forward: 5'- ATC AGA ACC ATC ATG GGC TGG ACA -3'
- Reverse: 5'-AGC CAA TCT TCT TCC AGA TGG TGA -3'

Mouse Bcl-2
- Forward: 5'- AAA GCC AGT GTT CCA TGC ACC AAG -3'
- Reverse: 5'-CAC ATG GCC GGC ACA CTT AAC ATT -3'

Mouse HO-1
- Forward: 5'-TCA GTC CCA AAC GTC GCG GT-3'
Reverse: 5’-GTC GTG CAG GRG TTG AGC C-3’

**Mouse 18S**

Forward: 5’- AGA AAC GGC TAC CAC ATC CAA-3’
Reverse: 5’- GGG TCG GGA GGT AAT TT -3’

**G. Western Blot analysis:**

Heart tissue homogenate (100 μg) was electrophoresed using SDS-PAGE method as previously described [99, 100]. An affinity purified rabbit anti-GAPDH antibody (1:3000) was purchased from Trevigen (Gaithersburg, MD) and p53 (1:1000) (Calbiochem) and HO-1 (1:1000) (Stressgen Biotechnologies) were detected using secondary antibodies.

**H. Cardiomyocyte isolation:**

Mouse cardiomyocytes were isolated from the whole heart by Liberase digestion as described previously with some modifications [101]. Briefly, mice were injected intraperitoneally with heparin (300 units) 30 min prior to sodium pentobarbital anesthetization (5 mg). The heart was rapidly excised, placed in
ice-cold PBS and the aorta was rapidly cannulated with a blunted, 22 gauge needle. The heart was then perfused in retrograde fashion with oxygenated Tyrode's solution maintained at 37°C to flush all blood from the coronary vasculature. After 5 minutes, the perfusion solution was exchanged with the Liberase digestion solution and the heart was digested for approximately 8 minutes. The ventricles were then cut off the cannula and placed in the mincing solution in which heart tissue was teased apart with forceps and tritutated using a serological pipette until cardiomyocytes were fully dispersed. Cardiomyocytes were passed through a 100 mesh filter to remove any undigested material, an allowed to settle to aspirate most of the mincing solution. The cardiomyocytes were suspended in re-suspension solution 1 and transferred to a 15mL tube and allowed to settle for 10 minutes. This solution was aspirated, along with any unsettled, hypercontracted cardiomyocytes and replaced with re-suspension solution 2. This process was repeated 5 times with increasing [Ca++] until only rod-shaped cardiomyocytes remained. The cell pellet was then resuspended in culture media and cells were plated on laminin-coated 37mm cell culture dishes and incubated at in a 5% CO₂ incubator at 37°C [101].
I. **Cardiomyocyte treatment:**

Once plated, and at the appropriate confluence, cells were incubated with Culture media (500 mL MEM (GIBCO), 5mL Insulin-transferrin, selenium (GIBCO), 1% pen-strep, glutamine 2mM, NaHCO3 4mM, HEPES 10mM, BSA 0.2%, BDM 1mg/ml) for 24 h. The cells were then treated with MeOH or 2.5 and 5 μM cLA and nitrite [85].

J. **Statistics:**

Data are shown as mean ± SEM. The n values represent the number of independent experiments. Each experiment was performed in triplicate unless otherwise indicated. P values were calculated by Student’s t-test or by one-way or two-way ANOVA, followed by Bonferroni analysis using GraphPad Prism software. A P value <0.05 was considered statistically significant.
CHAPTER IV

SPECIFIC AIM 1

**Specific aim 1**: To determine whether the co-treatment with cLA and nitrite protects heart function in mice following MI.

*Hypothesis*: Co-treatment with cLA and nitrite reduces LV volume and diameter in mice following MI.
Figure 1: Schematic diagram of overall hypothesis. Co-treatment with cLA and nitrite may enhance levels of HO-1 in heart tissue, leading to regulation of miRNA-499 and thus protecting heart from failure through induction of myocyte apoptosis.
Introduction

MI, a result of coronary artery occlusion, is a major healthcare problem within the United States [4]. According to the CDC, the most common type of heart disease is coronary artery disease, which commonly leads to MI, approximately 720,000 people annually within the United States present clinically with MI, and the subsequent costs exceed $108.9 billion. Current therapies for coronary artery disease are mostly preventative measures, such as healthy diet, exercise, and weight loss, highlighting the need for further research into effective therapies for MI. MI results from blockage of a coronary artery leading to insufficient blood supply to cardiac tissue, ultimately resulting in cardiomyocyte death [15] and cardiac dysfunction [13].

cLA is an 18:2 fatty acid isomer of linoleic acid and the cis-9 trans-11 isomer of cLA is the preferential fatty acid substrate for nitration, where nitration occurs only at positions 9 and 12 in vivo [86]. Co-treatment of cLA and nitrite in mice leads to an increase in nitrated-cLA within plasma and urine.

This specific aim examines the effects of cLA with and without nitrite supplementation on cardiovascular injury following MI. Considering the initiative to replace saturated fats with so-called ‘healthy fats’, to promote overall weight
loss and maintenance, the health effects of fats such as cLA require investigation. The principal findings demonstrated here, are that cLA is not protective in MI, but instead worsens survival after MI (Fig. 4). However, supplementation with nitrite in cLA-(10 mg/kg/d-via osmotic mini-pump) treated mice, leads to cardioprotection in MI injury. Specifically, cLA and nitrite co-treatment significantly reduces LV dilatation (Fig. 6-8) after MI and improves heart function (Fig. 6-7).

Atrial natriuretic peptide (ANP) is released from myocardial cells in the atria and in some cases the ventricles in response to volume overload and conceivably increased wall stress [102]. Brain natriuretic peptide (BNP) is initially identified in the brain but released primarily from the heart, particularly the ventricles [103]. The release of both ANP and BNP is increased in heart failure (HF), as ventricular cells are recruited to secrete both ANP and BNP in response to the high ventricular filling pressures [103].

4-HNE is a major and toxic aldehyde generated by free radical attack on ω-6 polyunsaturated fatty acids (arachidonic, linoleic, and linolenic acids) [104] and is considered a toxic messenger of oxygen free radicals [102, 103]. 4-HNE undergoes many reactions with various proteins, peptides, phospholipids, and nucleic acids; 4-HNE has a high biological activity and displays numerous cytotoxic, mutagenic, genotoxic, and signal effects [102, 103, 105], including inhibition of protein and DNA synthesis, inactivation of enzymes, modulation of platelet aggregation, and variety of the expression of numerous genes [104, 105].
In addition, 4-HNE may be an important mediator of oxidative stress–induced apoptosis [104], cellular proliferation, and signaling pathways. cLA and nitrite supplementation ameliorates MI induced oxidative stress and supports cardioprotection in this MI model (Fig. 9). Overall, cLA and nitrite mediate protection and result in improved heart function following MI.
Results:

1.1 ST-segment elevation is an early marker of MI

ECG was recorded during surgery, Fig. 3 A was recorded before surgery; Fig. 3 B was recorded after MI surgery. ST-segment elevation was recorded as an early sign of MI. After persistent ST-segment elevation on ECG, mouse chest was closed and moved to cage for recovery.

1.2 Combination treatment with cLA and nitrite improves survival in MI mice

Co-treatment of cLA and nitrite increased survival following MI. Survival in MI mice without treatment was between 70-80% and decreased to 35-40 % with cLA treatment. Survival following MI was improved with co-administration of cLA and nitrite (Fig. 4).

1.3 Co-administration of cLA and nitrite increases heart weight and reduces LV volume

Co-treatment of cLA and nitrite increased heart weight following MI. Hearts isolated from MI (10 days) and treated mice were found to be enlarged, with ventricular dilatation caused to increase EDV (Table 1). The heart weight / tibia length ratio was significantly increased in MI and single treated mice compared to control hearts, where co-administration cLA and nitrite was significantly higher than MI hearts with or without single treatment (Fig. 5).
1.4 Co-treatment with cLA and nitrite improve LV function following MI

MI is characterized by ventricular chamber dilation and dysfunction (Table 1). We observed a significant rightward shift of the PV loop in MI and single treated mice (Fig. 7). Chamber dilation also resulted in significant increase in both end-systolic and end-diastolic volume (Fig. 7, Table 1). As shown in Fig. 7, invasive hemodynamic measurements showed a reduction in EF in MI vs. the control groups (Fig. 7, Table 1). Treatment did not change EF in control animals (Fig. 7, Table 1). However, co-administration with cLA and nitrite shifted PV-loop to the left compared to MI PV-loop, indicating that the overall heart function is improved in cLA and nitrite-treated mice following MI.

Ultrasound showed similar changes in MI mice. LV end diastolic and end-systolic diameters were increased in mice, which caused LV Fraction Shortening (FS) reduction (Fig. 6). Treatment with cLA and nitrite improved LV function. FS was improved from 23% to 42% (Fig. 6).

1.5 Co-administration of cLA and nitrite eliminates MI induces oxidative stress in LV ventricle after MI

4-HNE is an indicator of lipid peroxidation and protein damage, and is considered to be a second toxic messenger of ROS [106]. There was brown pigmentation in tissue section from MI mice, which was a stain marker of 4-HNE-positive cells (Fig. 8). Brown spots were ameliorated in mice treated with cLA and
nitrite, meaning oxidative stress was reduced in mice treated with cLA and nitrite following MI (Fig. 8).

1.6 Co-treatment with cLA and nitrite eliminates apoptosis in ischemic zone of MI

TUNEL staining was used to detect apoptosis in LV of control, MI mice treated with or without cLA and nitrite. Apoptosis was detected in MI and single treated MI mice on ischemic part of LV (Fig. 9). Apoptosis was not visible in MI and single treated MI mice on non-ischemic part of LV (Fig. 9). Apoptosis was eliminated in MI mice treated with cLA and nitrite on ischemic zone of LV (Fig. 9). TUNEL staining did not stain the tissue from control mice (Fig. 9).

1.7 Co-administration of cLA and nitrite decreases ANP and BNP- markers for heart failure after MI

MI drastically increased ANP and BNP expression in heart, molecular markers of heart failure, by 4.2 and 3.4 fold, respectively (Fig. 10). However co-administration of cLA and nitrite eliminated ANP and BNP expression following MI (Fig. 10). Single treatment did protect heart from failure, as well as heart failure markers, ANP and BNP were increased by 4.2 and 2.9 fold respectively (Fig. 10).
Figure 2: Experimental study design. Mice were treated with cLA, nitrite, and cLA and nitrite 3 days prior to MI surgery. On day 3, MI surgery was induced and after 10 days PV-loop analysis was performed. Mice were killed and tissue was collected for additional analysis.
Figure 3: ST segment elevation is an early marker of MI. ECG was recorded during MI surgery. A. ECG recorded before the MI surgery, B. ECG recorded during the MI surgery. C. Example of ST segment elevation. ST-segment elevation was the marker of successful coronary artery ligation. After ST-segment elevation on ECG, the chest was closed and mouse was placed on a warming pad in the home cage for recovery.
FIGURE 4

Figure 4: Co-treatment with cLA and nitrite increases survival in mice following MI. Survival was increased in cLA and nitrite treated mice following MI compare to single treated MI mice (*p<0.05 to MI cLA N, n=24 per group).
Figure 5: Co-treatment with cLA and nitrite increases heart weight following MI. Heart weight was significantly increased following cLA or nitrite following MI, or in MI alone. cLA and nitrite co-treatment resulted in a further increase in heart weight, which was significant compared to MI alone. (*p<0.05 to control, #p<0.05 to MI, n=24 per group)
Figure 6: Co-treatment with cLA and nitrite improves LV fraction shortening in mice following MI. A and D illustrate control mouse heart in B-mode and M-mode respectively. B and E illustrate MI mouse heart in B-mode and M-mode respectively. C and F illustrate MI mouse heart treated with cLA and nitrite in B-mode and M-mode respectively. G illustrates formula - how to calculate FS.

\[ FS = \frac{LVEDd - LVESd}{LVEDd} \]

(*p<0.05 to control, #p<0.05 to MI, n=24 per group)
FIGURE 7

Figure 7: Co-treatment with cLA and nitrite significantly improves cardiac function after MI. Treatment with cLA exacerbated cardiac injury after MI, where combination treatment (supplementation with nitrite) rescued heart function following MI. (*p<0.05 to control, #p<0.05 to MI, n=10 per group).
Figure 8: Co-treatment with cLA and nitrite reduces oxidative stress following MI. **A.** represents control heart tissue, where 4-HNE-brown pigment was not expressed. **B, C, and D** represent tissue from MI and single treated mice with cLA or nitrite, brown pigment stain-4-HNE was highly expressed in those samples. **E** represents tissue from the MI mouse heart, which was treated with cLA and nitrite, brown pigmentation-4-HNE was eliminated in this sample. **F** represents quantification using Adobe Photoshop. (*p<0.05 to control, #p<0.05 to MI, n=10 per group).
Figure 9: Co treatment with cLA and nitrite attenuates myocyte apoptosis following MI. Apoptosis was detected on tissue using TUNEL staining. Pictures were collected from ischemic zone (IZ) and from non-ischemic zone (NIZ) in MI mice. A represents confocal images from experimental groups, and B represents quantification using Adobe Photoshop. (*p<0.05 to control, n=6 per group).
**FIGURE 10**

Figure 10: Co-treatment with cLA and nitrite ameliorates heart failure markers following MI. relative fold changes of (A) ANP and (B) BNP expression in mouse hearts. ANP- atrial natriuretic peptide; BNP- B-type natriuretic peptide.

(*p<0.05 to control, #p<0.05 to MI; n=6 per group).
Table 1: Hemodynamics of MI and control mice based on pressure-volume. All values are expressed as mean ± SD. BPM – beats per minute; ESP – end-systolic pressure; EDP – end-diastolic pressure; ESV – end-systolic volume; EDV- end-diastolic volume, SV – stroke volume; CO – cardiac output; EF – ejection fraction; (*p<0.05 to control, #p<0.05 to MI, n=10 per group).

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Discussion

Here we examine the effects of cLA with and without nitrite supplementation on cardiovascular injury following MI. Considering the initiative to replace saturated fats with so-called ‘healthy fats’, to promote overall weight loss and maintenance, the health effects of fats such as cLA require further investigation. The principal findings demonstrated here, are that cLA is not protective in MI, but instead worsens survival following MI (Fig. 4). However, supplementation with nitrite in cLA (10mg/kg/d-via osmotic mini-pump)-treated mice, leads to improved survival in mice following MI (Fig. 4). Specifically, cLA and nitrite co-treatment significantly decreases MI induced ANP and BNP mRNA levels following MI (fig. 10). Co-treatment with cLA and nitrite also decreases oxidative stress - 4-HNE expression following MI (Fig. 8). Overall, cLA and nitrite mediate protection and result in improved heart function and reduced LV volume in this murine model of MI (F. 6,7 and Table 1).

Previous studies reported the correlation between hemodynamics and neurohumoral factors such as plasma cardiac natriuretic peptides, and plasma levels of ANP and BNP were thought to be a hemodynamic predictor in patients with CHF [105]. It is well known that plasma levels of ANP and BNP are markedly increased in patients with acute MI as well as in those with congestive heart failure [107, 108]. Our study shows increased ANP following MI and in single treated mice, which was lowered in cLA and nitrite treated mice (Fig. 10). Our physiological data demonstrates similar correlation with ANP and BNP, it shows
LV dilatation in MI and single treated mice, LV volume and diameter is decreased in cLA and nitrite treated MI mice (Fig 7, Table 1).

A prominent lipid peroxidation product, 4-HNE, can be cytotoxic and is generated during various physiological and pathophysiological conditions based on the generation and cellular location of ROS [109, 110]. 4-HNE is also known as a measure of oxidative injury [111, 112]. Consequently, 4-HNE has biological relevance and displays a number of cytotoxic, mutagenic, genotoxic and further signal effects [113, 114]. Our data shows that 4-HNE was increased in MI and MI treated with cLA or nitrite mice, however oxidative stress was reduced after combination treatment with cLA and nitrite. cLA and nitrite supplementation ameliorates MI induced oxidative stress and supports cardioprotection in this MI model (Fig. 8). Overall, cLA and nitrite mediate protection and result in improved heart function following MI.

Many studies have shown that TUNEL staining of nuclei to conclude that apoptosis occurs following MI [115]. The presence of apoptotic nuclei suggested that apoptosis was present in all cells in acute MI as a primary form of cell death [42]. LV remodeling during MI is characterized by progressive LV chamber dilation and systolic and diastolic dysfunction [116-118]. The LV remodeling involves cellular and molecular mechanisms at the site of infarction and the surviving unaffected area [119]. Myocyte apoptosis occurs in the ischemic zone of myocardium post MI [15, 34]. The amount of apoptotic myocytes correlates with an increase in the ventricular diameter after MI [119]. Apoptosis in the
infarcted area of myocardium plays a big role in LV remodeling after MI in mice [99]. Overall our study demonstrates that apoptosis was increased in MI and single treated MI mice, based on TUNEL staining of nuclei, which was reduced after combination treatment with cLA and nitrite (Fig. 9). As observed in our studies where we measured LV volume, TUNEL staining was reduced along with reduction of LV size and volume (Fig. 9, Table 1).

*Based on our data we conclude that treatment with cLA and nitrite reduces LV volume and LV size after MI, as well as reduces oxidative stress and attenuates apoptosis in ischemic area of myocardium after MI.*
CHAPTER V

SPECIFIC AIM 2

**Specific aim 2:** To determine whether co-treatment with cLA and nitrite increases HO-1 level in cardiac tissue, thereby increasing miRNA-499 and inhibiting p53-induced apoptosis.

*Hypothesis:* HO-1 expression increases miRNA-499 and provides a cardioprotective effect.
Figure 11: Schematic diagram of specific aim 2 hypothesis. Co-treatment with cLA and nitrite may enhance levels of HO-1 and/or miRNA-499 in heart tissue, leading to regulating oxidative stress and thus protecting heart from failure through induction of myocyte apoptosis.
Introduction

MI is characterized by significant changes in gene expression, many of which represent adaptive or maladaptive responses to stress [16-20]. The resulting cardiac stress induces rapid changes in gene expression immediately following MI [21]. Cardiomyocyte cell death is a consequence of myocardial injury, which occurs as early as the initiation of acute MI [22]. Cardiomyocyte death or apoptosis is a key factor in transition from cardiac hypertrophy to heart failure [23]. p53 is a well-known transcription factor which mediates apoptosis by activating the manifestation of pro-apoptotic genes [96]. Previous studies have demonstrated that p53 activity is enhanced during MI and that p53 plays a critical role in the regulation of hypoxia-induced apoptosis of cardiomyocytes [1, 40]. Thus, p53 becomes an important therapeutic target, with regard to attenuation of cardiac apoptosis and consequent heart failure.

Apoptosis is controlled by several genes and the most crucial regulators are members of the Bcl-2 gene family. Bcl-2 is an anti-apoptotic gene that was first identified in follicular non-Hodgkin lymphoma [102, 104]. It has the capability to block an inclusive selection of apoptotic signals [103]. Bax is another member of the Bcl-2 family, but compared to Bcl-2 it has an apoptosis-stimulating function [105]. Gene products of the Bcl-2 family can form homo- and heterodimers with
each other [107]. Therefore, it has been proposed that the cellular Bcl-2/Bax ratio is a key factor in the regulation of apoptosis; a high Bcl-2/Bax ratio makes cells resistant to apoptotic stimuli, while a low ratio induces cell death [108]. The Bax to Bcl-2 ratio is increased in cells induced to die by an apoptotic stimulus [107, 108]. Therefore, the overexpression of Bax after MI may be related to the pathogenesis of apoptosis and congestive heart failure following MI.

MicroRNAs quantitatively regulate mature-RNAs, which affect the cardiac transcriptional output and cardiac function [55]. miRNAs are endogenous, single-stranded non-coding RNAs ranging from 18 to 24 nucleotides in length, that play an important role in gene regulation [55, 56]. In screening for miRNAs enriched in the heart, we found an abundant miRNA, miRNA-499, which is an evolutionarily conserved muscle-specific microRNA that is encoded within the intron of myosin heavy chain and is highly expressed in the cardiac ventricles [55-57]. Plasma miRNA-499 is known as a biomarker of acute MI, as plasma miRNA-499 has been observed in individuals with MI [58].

Studies have shown that diets with diversified fats may be an effective strategy in decreasing risk of cardiovascular disease (CVD) [120, 121]. Conjugated linoleic acid (cLA) is a fatty acid that occurs as a mixture of positional and geometric isomers of linoleic acid (LA), which is produced in ruminant animals via an enzymatic isomerase reaction [122]. cLA contains 18 carbon atoms with two conjugated double bonds separated by a single bond [123]. cLA is found naturally in food products from these animals predominantly as the cis-9,trans-11 form, whereas synthetic cLA preparations consist of a few different
isomers with an approximately equal amount of cis-9,trans-11 and trans-10,cis-12 cLA [121, 124]. Select modified fatty acids, such as nitrated fatty acids are known to induce pluripotent anti-inflammatory effects [125]. Recent literature suggests that dietary cLA and nitrite supplementation in rodents elevates NO₂-cLA levels in the plasma and tissues, inducing heme oxygenase-1 (HO-1) expression in the target tissue [86]. HO-1 catalyzes the oxidation of heme-producing biliverdin, iron, and carbon monoxide (CO) [87, 88]. Importantly, biliveridin is converted to bilirubin, a known potent antioxidant. Additionally, iron can be sequestered by ferritin, leading to off target anti-apoptotic effects and carbon monoxide has similar characteristics to nitric oxide, facilitating numerous biological functions including anti-inflammatory effects [88-90].

Ultimately, heart function is highly dependent on ATP generation. The heart is enriched with mitochondria that provide the energy required for cardiomyocyte function [62]. Therefore, it is known that mitochondria play a critical role in the development and progression of many cardiac diseases such as hypertrophy and MI. Previous studies suggest that mitochondria are highly dynamic and that changes in mitochondrial shape can affect a variety of biological processes such as apoptosis and fibrosis [63, 64]. Mitochondria are dynamic organelles, which constantly undergo fission and fusion [63-65]. These two opposing processes are regulated by the mitochondrial fusion proteins mitofusin and the mitochondrial fission protein Drp-1 [62]. During apoptosis, Drp-1 foci accumulates on mitochondria and can enhance mitochondrial fission [66, 67].
In the present study an in vivo model of MI is used to further elucidate the possible mechanism whereby combination treatment with cLA and nitrite is cardioprotective. Herein we demonstrate that cLA and nitrite significantly induced levels of miRNA-499 in the heart compared to untreated MI mice. Additionally, co-treatment significantly reduced levels of p53 expression and induced expression of HO-1. These results suggest that treatment with cLA and nitrite may provide significant protection during MI through regulation of cardiac specific miRNA and upregulation of phase 2 antioxidant enzyme expression.
Results:

2.1 miRNA-499 biomarker of acute MI

miRNA-499 was measured in isolated hearts from MI and control mice after 24h and 10 days after MI (Fig. 12). In the control, miRNA-499 almost had undetectable concentrations. Compared to the healthy control mice, miRNA-499 levels were higher in MI mice after 24h (Fig. 12). Furthermore, the level of miRNA-499 was lowered in MI mice after 10 days compare to MI 24h mice (Fig. 12).

2.2 Co-treatment of cLA and nitrite regulates miRNA-499 during MI

To investigate the specific role of miRNA-499 in regulation of improved heart function following MI, miRNA-499 levels were measured in the heart (Fig. 13). miRNA-499 was increased following MI with combination treatment of cLA and nitrite (Fig. 13). There were no significant differences in single treated MI mice or with MI alone (Fig. 13).

2.3 Mitochondrial fission factor DRP-1 is significantly increased in mice following MI

MI caused a robust increase in Drp-1 expression in MI (Fig. 14). Individual treatments with cLA or nitrite increased expression of Drp-1 compared to control
mice, but the expression was overall lower than with MI-induction (Fig. 14). Co-treatment with cLA and nitrite attenuated Drp-1 expression in MI mice (Fig. 14).

2.4 cLA and nitrite treatment attenuates p53 expression following MI

Expression of p53 was defined by immunohistochemical staining of cryo-sectioned hearts (Fig. 15A). To support the immunohistochemical results that miRNA-499 blocks p53 after co-administration treatment with cLA and nitrite during MI, the level of p53 was assessed by Western blot analysis (Fig. 15B). The protein level of p53 was increased following MI and in single treated mice, but was abolished after co-treatment with cLA and nitrite (Fig. 15B). The p53 staining support the data obtained via Western blot analysis (Fig. 15B).

2.5 HO-1 expression increases in MI following cLA and nitrite treatment

HO-1 expression was increased, as demonstrated using immunohistochemical staining of cryo-sectioned heart tissue (Fig. 15A). To support the hypothesis that co-treatment with cLA and nitrite during MI induces HO-1 level, the expression of HO-1 was assessed using Western blot analysis (Fig. 15B). Following treatment with cLA and nitrite, HO-1 protein expression is significantly increased (Fig. 15B).
2.6 Co-treatment with cLA and nitrite attenuates pro-apoptotic markers following MI

The total Bax mRNA level was increased and the Bcl-2 mRNA level was decreased in the MI and single treated MI mice leading to an induction in the Bax/Bcl-2 ratio (Fig. 16). mRNA level of Bax was increased compared to control, levels were not as high as in MI and single treated MI mice (Fig. 16) and mRNA of Bcl-2 was not changed in cLA and nitrite treated MI mice. Ratio of Bax/Bcl-2 was induced in co-treated MI mice, but was lower than in MI and single treated mice (Fig. 16).

2.7 Induction of HO-1 attenuates LV dysfunction in MI mice

As we mentioned before, MI was characterized by ventricular chamber dilation and dysfunction (Table 2). We observed a significant rightward shift of the PV loop in MI mice (Fig. 18). Chamber dilation also resulted in significant increase in both end-systolic and end-diastolic volume (Fig. 18, Table 2). As shown in Fig. 8, invasive hemodynamic measurements showed a reduction in %EF in MI vs. the control groups (Fig. 18, Table 2). HO-1 Tg mice had decreased %EF compared to control mice (Fig. 18, Table 2). However, %EF in HO-1 Tg mice was higher than in WT MI mice (Fig. 8).
Ultrasound showed similar changes in MI mice. LV end diastolic and end-systolic diameters were increased in mice, which caused LV Fractional Shortening (FS) reduction (Fig. 19). %FS in HO-1 Tg mice was improved following MI. FS was improved from 23% to 47% (Fig. 19).

2.8 HO-1 attenuates p53 levels in HO-1 Tg mice following MI

HO-1 protein level was greater in HO-1 Tg mice compared to C57 control mice (Fig. 20). As we demonstrated previously, HO-1 protein level was high in MI mice treated with cLA and nitrite. HO-1 protein level expression stayed high in HO-1 Tg mice compared to control HO-1 Tg control mice (Fig. 20). HO-1 protein level was not expressed in HO-1 KO mice regardless of treatment (Fig. 20).

From the previously demonstrated blot we saw that p53 protein level was high in C57 mice compared to control. We demonstrated the same result in Figure 20. From the same blot we saw that p53 protein level was drastically increased in HO-1 KO control, HO-1 KO MI and HO-1 KO MI mice treated with cLA and nitrite (Fig. 20). The protein level was low in HO-1 Tg Control and MI mice (Fig. 20).
2.9 HO-1 induces miRNA-499 expression in mice following MI.

To investigate the relationship between HO-1 and miRNA-499 we measured miRNA-499 level in HO-1 Tg and KO mice following treatment and MI surgery. miRNA-499 was high in HO-1 Tg control mice and was further increased after MI in HO-1 Tg mice (Fig. 21). miRNA-499 level was not expressed in HO-1 KO mice regardless of treatment or surgery (Fig. 21).

2.10 HO-1 attenuates ratio of Bax/Bcl-2 following MI

The total Bax mRNA level was increased and the Bcl-2 mRNA level was decreased in the HO-1 KO mice regardless of the treatment (Fig. 22). mRNA level of Bax was increased in HO-1 KO mice compared to control, levels were higher compared to C57 MI mice (Fig. 22) and mRNA of Bcl-2 was low in HO-1 KO mice (Fig. 22). Ratio of Bax/Bcl-2 was induced in HO-1 Tg control and MI mice (Fig. 22).

2.11 The heart failure markers (ANP and BNP) are lowered in HO-1 Tg mice after MI.

To evaluate heart failure in HO-1 Tg and HO-1 KO mice after MI we measured expression of ANP and BNP (Fig. 23). Expression of ANP and BNP were markedly increased in C57 MI mice, those markers were farther increased
in HO-1 KO mice, which was 5 fold higher than in C57 MI mice (Fig. 23). ANP and BNP expression was not changed in HO-1 Tg mice after MI (Fig. 23).

2.12 cLA and nitrite induces miRNA-499 and HO-1 expression in cardiomyocytes.

For further investigation of the relationship between treatment miRNA-499 and HO-1 level expression, we isolated myocytes from adult C57 mice and treated with two different doses of cLA and nitrite (Fig. 25). HO-1 and miRNA-499 expression was increased in cardiomyocytes treated with 2.5uM cLA and nitrite (Fig. 25). The expression of both HO-1 and miRNA-499 was further increased in cardiomyocytes treated with 5uM cLA and nitrite (Fig. 25).

2.13 cLA and nitrite induces miRNA-499 through HO-1 induction in cardiomyocytes

We investigated the direct relationship between HO-1 and miRNA-499 using HO-1 Tg and HO-1 KO mice; we isolated myocytes and treated with cLA and nitrite. Expression of HO-1 was very high in HO-1 Tg control isolated cardiomyocytes compared to C57 control isolated cardiomyocytes (Fig. 27). Expression of HO-1 was increased in HO-1 Tg isolated cardiomyocytes treated with 5uM cLA and nitrite (Fig. 27). HO-1 was not expressed in HO-1 KO isolated cardiomyocytes regardless of treatment (Fig. 27). Expression of miRNA-499 was
similar in HO-1 Tg mice. It was highly expressed in HO-1 Tg control isolated cardiomyocytes compared to C57 control isolated cardiomyocytes (Fig. 27). It was further increased in HO-1 Tg cardiomyocytes treated with 5uM cLA and nitrite mice (Fig. 27).
Figure 12: miRNA-499 biomarker of acute MI. miRNA-499 was drastically increased after 24h of MI compared to control mice, which was lowered after 10 days of MI compared to 24h MI mice. (*p<0.05 vs Control; # p<0.05 vs MI 24h n=6 per group)
Figure 13: Co-treatment with cLA and nitrite induces miRNA-499 following MI. miRNA-499 was significantly increased in MI mice after combination treatment with cLA and nitrite (*p<0.05 vs control, n=8 per group).
Figure 14: Mitochondrial fission factor DRP-1 is attenuated in mice treated with cLA and nitrite following MI. Nitrite or cLA-treated MI mice had decreased levels of DRP-1 compared to MI alone. Combination treatment with cLA and nitrite significantly lowered the level of Drp-1 in MI mice, comparable to control values (*p<0.05 to control, #p<0.05 to MI, n=8 per group).
Figure 15 A: Co-treatment with cLA and nitrite attenuates p53 and induces HO-1 expression following MI.
Figure 15 B: Co-treatment with cLA and nitrite attenuates p53 and induces HO-1 expression following MI. Confocal microscopy (A): heart tissue was labeled with p53 (green), HO-1 (red), and cell nuclei were labeled with DAPI (blue). Western blot analysis reveals that p53 is significantly increased in cardiac tissue following MI (B). Quantitated protein expression reveals that co-administration of cLA and nitrite lowers p53 levels, while increasing expression of HO-1 (*p<0.05; n=6 per group).
Figure 16: Co-treatment with cLA and nitrite attenuates pre-apoptotic markers following MI. Expression of Bcl-2 and Bax in mice with/or without treatment following MI. (A-C) mRNA levels of Bcl-2 and Bax. (A) Higher Bax was observed in MI and single treated mice, which was lowered in cLA and nitrite treated mice (*p=0.05 vs control; #p=0.05 vs MI, N=6) and (B) lower Bcl-2 was observed in MI and single treated mice, which was restored in MI mice treated
with cLA and nitrite (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group). (C) Ratio of Bax/Bcl-2 was high in MI and single treated mice which were lowered in MI mice treated with cLA and nitrite (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group). (D) Schematic representation of relationship between Bax and Bcl-2.
Overexpression of HO-1 increased levels of miRNA-499 in heart after MI, resulting in inhibition of p53 and an overall improvement of heart function in MI mice.
Figure 18: Induction of HO-1 attenuates LV dysfunction following MI. MI causes a drastic increase of End-diastolic volume in C57 mice (pink lines), which was protected in HO-1 Tg mice (red lines) compared to control (black lines). EF of C57 MI mice was decreased, 23%, HO-1 Tg mice EF-47% was decreased compared to control mice (EF-62%) but it was not as low as C57 MI mice. (*p<0.05 to control, #p<0.05 to MI, n=10 per group)
Figure 19: Fraction shortening is protected in HO-1 Tg mice following MI.

FS was decreased in C57 mice- 23% compared to control C57 mice-52%. FS of HO-1 Tg mice- 47% was lower compared to C57 control mice-52%, but was higher than in C57 MI mice-23%. (*p<0.05 to control, #p<0.05 to MI, n=24 per group)
Figure 20: MI does not alter HO-1 levels in HO-1 Tg mice following MI. Examples of Western Blot images of the protein studied and contents of GAPDH in the respective samples (A). Relative protein expression is reported as fold change (B), alone (*p<0.05 to control, #p<0.05 to MI, n=24 per group)
Figure 21: HO-1 induces miRNA-499 expression in mice following MI. The bar graph represents real-time PCR amplification of miRNA-499. miRNA-499 was highly expressed in MI mice treated with cLA and nitrite and in HO-1 Tg mice with or without MI, miRNA-499 was not expressed in HO-1 KO mice regardless of treatment. (*p<0.05 to control, #p<0.05 to MI, n=6 per group)
Figure 22: HO-1 attenuates apoptosis following MI. Expression of Bcl-2 and Bax in C57, HO-1KO and HO-1Tg mice with/or without treatment following MI. (A-C). Higher Bax was observed in C57 MI and HO-1 KO mice (A), which was lowered in cLA and nitrite treated mice and HO-1 Tg mice (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group). Lower Bcl-2 was observed in C57 MI and HO-1 KO mice, which was restored in MI mice treated with cLA and nitrite and HO-Tg mice (B) (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group). (C) Ratio of
Bax/Bcl-2 was high in C57 MI and HO-1 KO mice which were lowered in MI mice treated with cLA and nitrite and HO-1 Tg mice (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group). (D) Schematic representation of relationship between Bax and Bcl-2.
Figure 23: HO-1 reduces heart failure markers after MI. Relative fold change of (A) ANP and (B) BNP in C57, HO-1 KO, and HO-1 Tg mice before and after MI with/without treatment with cLA and nitrite. ANP-atrial natriuretic peptide; BNP- B-type natriuretic peptide. (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group).
Figure 24: Schematic diagram of specific aim 2 hypothesis.

Co- treatment with cLA and nitrite induces level of HO-1 in myocytes.
Figure 25: cLA and nitrite induces miRNA-499 and HO-1 expression in mice following MI. HO-1 expression was increased by 4 fold after 2.5\(\mu\)M cLA and nitrite treatment. 5\(\mu\)M cLA and nitrite administration caused a 15 fold increase of HO-1 expression in C57 myocytes (A). miRNA-499 expression was increased for 10 fold after 2.5\(\mu\)M cLA and nitrite treatment. 5\(\mu\)M cLA and nitrite administration caused more than a 22 fold increase of miRNA-499 expression in C57 myocytes (B). (\(*\)p=0.05 vs control; \#p=0.05 vs MI, n=6 per group).
FIGURE 26

Figure 26: Schematic diagram of specific aim 2 hypothesis. HO-1 is regulating the expression of miRNA-499.
Figure 27: cLA and nitrite induces miRNA-499 level through HO-1. HO-1 expression was high before cLA and nitrite treatment in HO-1 Tg cardiomyocytes, HO-1 expression was increased further after 5μM cLA and nitrite administration (A). miRNA-499 expression was high in HO-1 Tg cardiomyocytes before treatment, which was increased further after 5μM cLA and nitrite administration (B). (*p=0.05 vs control; #p=0.05 vs MI, n=6 per group).
### Table 2

<table>
<thead>
<tr>
<th></th>
<th>HR (BPM)</th>
<th>ESP (mmHg)</th>
<th>EDP (mmHg)</th>
<th>ESV (µL)</th>
<th>EDV (µL)</th>
<th>SV (µL)</th>
<th>CO (µl/min)</th>
<th>EF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 control</td>
<td>552±1.788</td>
<td>80.87±0.488</td>
<td>6.94±0.869</td>
<td>15.43±0.389</td>
<td>40.66±1.722</td>
<td>25.23±1.533</td>
<td>13926.9±449.898</td>
<td>82.04±2.127</td>
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<tr>
<td>C57 MI</td>
<td>555.77±6.673</td>
<td>78.36±3.719</td>
<td>2.73±1.247</td>
<td>55.43±3.719</td>
<td>72.77±2.396</td>
<td>17.65±3.716</td>
<td>9800.19±1218.053</td>
<td>23±4.168</td>
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<tr>
<td>HO-1 Tg MI</td>
<td>545.72±3.501</td>
<td>80.87±1.344</td>
<td>0.11±2.164</td>
<td>29.87±2.344</td>
<td>55.17±1.538</td>
<td>26.15±4.846</td>
<td>13767.5±121353</td>
<td>47.38±6.620</td>
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**Table 2:** Hemodynamics of C57 control, C57 MI and HO-1Tg MI mice based on pressure-volume. All values are expressed as mean ± SD. BPM – beats per minute; ESP – end-systolic pressure; EDP – end-diastolic pressure; ESV – end-systolic volume; EDV – end-diastolic volume, SV – stroke volume; CO – cardiac output; EF – ejection fraction; (*p<0.05 to control, #p<0.05 to MI, n=10 per group).
Discussion

This thesis examines the effects of cLA with and without nitrite supplementation on cardiovascular injury following MI. Considering the initiative to replace saturated fats with so-called ‘healthy fats’, to promote overall weight loss and maintenance, the health effects of fats such as cLA require investigation. The principal findings demonstrated here is that cLA and nitrite co-treatment significantly increases miRNA-499 and blocks mitochondrial fission through inhibition of p53 (Fig. 13, 14 and 15). Further, co-treatment with cLA and nitrite also induces HO-1 protein expression (Fig. 15), which supports cardio-protection in this MI model. Overall, cLA and nitrite mediate protection and result in improved heart function in this murine model of MI.

It is known that mitochondria undergo fission and fusion events continuously in non-disease states [126]. Mitochondrial morphological dynamics affect the outcome of ischemic heart damage and pathogenesis [126]. The Drp1 protein plays an important role in fission, regulating mitochondrial membrane dynamics [65, 126, 127]. Drp1 exists as small oligomers, located primarily at the mitochondrial outer membrane. These oligomers can connect to each other, forming larger multimeric structures, thus mediating mitochondrial division [16, 65, 127, 128]. In previous studies Drp1 has been identified as a mediator of mitochondrial morphological changes and cell death during cardiac ischemic injury [128]. It has been reported that miRNA-499 affects DRP-1 mediated apoptosis and the severity of MI and cardiac dysfunction during heart disease
Our data suggest that MI significantly increases Drp1 expression in the heart of single treatment with cLA- and nitrite-treated, as well as in non-treated mice (Fig. 13). Drp1 expression was abolished following co-treatment with cLA and nitrite in MI mice (Fig. 13).

Bcl-2 gene families are identified as apoptosis regulating genes. Of these genes, Bax promotes cell death, whereas Bcl-2 inhibits apoptosis and promotes cell survival [108, 129]. The pro-apoptotic Bax gene shares structural similarities with Bcl-2 and is thought to inactivate Bcl-2 by binding to it [130]. The expression of Bax is dramatically increased in MI mice (Fig. 16), whereas a tendency toward a decrease in Bcl-2 expression is also revealed (Fig. 16). Although the threshold of Bax/Bcl-2 ratio at which apoptosis is triggered in the cardiac cell is not known, our data, together with others [131, 132], suggest that an increased Bax/Bcl-2 ratio increases the probability for a myocardial cell to undergo apoptosis following MI (Fig. 16). The role of other pro-apoptotic and anti-apoptotic genes needs further investigation.

There is evidence that miRNA-499 controls the apoptotic pathway thorough regulation of p53 [57]. Our data supports the hypothesis that miRNA-499 decreases Drp1 levels after combination treatment with cLA and nitrite (Fig. 11). MI is known to cause cardiomyocyte ischemia, in turn, leading to p53-dependent cardiomyocyte apoptosis [1, 2]. The current study extends previous findings by demonstrating that ischemia causes increased expression of p53 following MI in non-treated and cLA or nitrite-treated mice and provides
significant implications with regard to the molecular mechanism of cardiomyocyte apoptosis following MI (Fig. 15).

Previous studies have shown that pharmacological delivery of nitrated fatty acids leads to protection from cardiovascular injury [133, 134]. These studies have focused on protective signaling pathways including inhibition of p65 subunit of NFκB and increased expression of HO-1. In a model of stenosis femoral artery injury, nitrated oleic acid induced vascular expression of HO-1, mediating protection against neointimal hyperplasia [134]. Nitrated oleic acid inhibited activation of NFκB in a murine model of ischemia reperfusion injury, resulting in reduction of infarct size [133]. More recent data suggests that dietary cLA is a privileged substrate for nitration reactions facilitated by mitochondria, digestion, and macrophage activation and following metabolic stress such as MI [86]. Co-administration of dietary cLA and nitrite supplementation raises NO2, cLA levels in plasma and tissues, which in turn induces HO-1 expression in target organs [86].

In the failing heart, HO-1 has anti-apoptotic effects by attenuating cell loss, p53 expression, and pathological heart remodeling [90]. Our data suggest that ratio of Bax/Bcl-2 was low in HO-1 Tg mice (Fig. 22), and it was drastically increased in HO-1KO mice after MI. Treatment with cLA and nitrite could not protect HO-1 KO hearts from apoptosis (Fig. 22). Heart failure marker expression supported apoptosis markers and physiological data (Fig. 18 and 22). ANP and BNP was low in HO-1 Tg mice following MI (Fig. 23), while those markers were
severely high in HO-1 KO mice regardless of treatment with cLA and nitrite (Fig. 23).

The important novelty of this thesis was to answer to the question: does HO-1 overexpression increase miRNA-499 levels, resulting in inhibition of p53 level in heart tissue and an overall improvement of heart function in mice following MI (Fig. 17)?

To answer this question we used HO-1 Tg and HO-1 KO mice. As we mentioned before, there are many studies showing that induction of HO-1 resulted in cardiac protection during MI [90, 135-137]. Our data demonstrates similar results: MI in HO-1 Tg does not have drastic changes in LV function as in C57 mice (Fig. 18, 19 and Table 2). Western Blots supports the physiological data; HO-1 protein expression was markedly high in HO-1 Tg mice and was not expressed in HO-1 KO mice (Fig. 20), which explains the cardio-protective effect in HO-1 Tg mice, as their heart function was preserved compared to C57 MI mice (Fig. 18, 19 and Table 2).

As we demonstrate in our study cLA and nitrite co-administration decreased apoptosis though the HO-1 and/or miRNA-499 pathway. To investigate more deeply the role of HO-1 and/or miRNA-499 in apoptosis, we used HO-1 Tg and HO-1 KO mice. Our data supported the hypothesis that HO-1 regulates miRNA-499 levels and thus decreases apoptosis after MI. As others and we have demonstrated before, MI is known to cause cardiomyocyte ischemia, in turn, leading to cardiomyocyte apoptosis [1, 2]. The current study
extends previous findings by demonstrating that ischemia causes an increased ratio of Bax/Bcl-2 following MI in non-treated C57 mice and in HO-1 KO mice (Fig. 22). Our data demonstrate that in HO-1 KO mice the expression of miRNA-499 is not detectable (Fig. 21).

Treatment with cLA and nitrite increased expression of HO-1 in isolated cardiomyocytes from C57 and HO-1Tg mice (Fig. 25 and 26), as well as increased miRNA-499 level in isolated cardiomyocytes from C57 and HO-1 TG mice (Fig. 25 and 26). Data further suggests that co-administration of cLA and nitrite cannot increase the levels of HO-1 and meanwhile cannot change the levels of miRNA-499 in isolated cardiomyocytes from HO-1 KO mice (Fig. 25 and 26).

Overall, these data reveal mechanistic links between p53, HO-1, miRNA-499, and Drp1 with regard to regulation of the apoptotic programmed cell death in the heart. Taken together, these results suggest that therapeutic treatment with cLA and nitrite may provide cardiac protection during MI through the regulation of induction of cardiac specific HO-1 expression, which further regulated cardiac specific miRNA-499. Further validation studies are required in small and large preclinical animal models with the goal of clinical translation.
CHAPTER VI

IMPLICATION IN HEALTH AND DISEASE

Multiple clinical trials support that the increased dietary intake of unsaturated fatty acids promotes a broad range of physiological benefits [86, 138]. Results from Herbel reported a mean dietary cLA intake of 139 mg/d in young men and women [139], and Park’s lab showed an estimated mean cLA intake in lactating women during periods of low and high dairy consumption of 20 and 290 mg/d, respectively [140]. In summary, chronic as well as acute total cLA intake in men and women has not been reported to exceed 500 mg/d [141].

Dairy products were the primary source of total cLA, followed by beef [70, 73]. It has been identified that conjugated linoleic acid (cLA) is the preferential substrate for fatty acid nitration in humans [86]. Most importantly, it has been reported that cLA nitration occurs in healthy humans (as measured in plasma and urine) and may be increased in experimental models of inflammation upon oral delivery of cLA and inorganic nitrite (NO$_2$) [86]. Because of the public concern with nitrite toxicity (the lethal dose in humans is about 22 milligrams per kilogram of body weight), the maximum allowed nitrite concentration in meat
products is 200 ppm [142]. Approximately 80 to 90% of the nitrite in the average U.S. diet is not from cured meat products, but from natural nitrite production from vegetable nitrate intake [142]. In our lab we have demonstrated that in mice, nitrite consumption together with cLA administration prevents heart failure, which may be one explanation for the apparent health effect of the Mediterranean diet [143].
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Conduct clinical studies on drug effects and evaluate efficacy of the drug.


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PUBLICATIONS

1. Qipshidze N, Tyagi N, Sen U, Givvimani S, Metreveli N, Lominadze D, Tyagi SC. Folic acid mitigated cardiac dysfunction by normalizing the levels of tissue inhibitor of metalloproteinase and homocysteine-metabolizing enzymes. Post


5. **Qipshidze-Kelm N**, Piell KM, Solinger J, Cole MP. Combination Treatment with Conjugated Linoleic Acid and Nitrite Protects Against Myocardial Infarction. 10.1015/J.redox.2013.10.009

**as a Co-Author (Selected):**


18. Khundmiri SJ, Salyer SA, Farmer B, Qipshidze-Kelm N, Murray RD, Clark BJ, Xie Z, Pressley TA, Lederer ED. Structural determinants for the ouabain-stimulated increase in Na-K ATPase activity,

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**National / International Meetings**

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