2013

Molecular Mechanisms Related to Endotoxemia in Primary Human Cardiomyocytes in Culture

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Molecular Mechanisms Related to Endotoxemia in Primary Human Cardiomyocytes in Culture

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A Thesis Presented to Georgia Campus- Philadelphia College of Osteopathic Medicine

In Partial Fulfillment

For the Degree of

Master of Science

In

Biomedical Science

Georgia Campus- Philadelphia College of Osteopathic Medicine August 2013

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This thesis has been presented to and accepted by the Associate Dean for Curriculum and Research Office of Georgia Campus- Philadelphia College of Osteopathic Medicine in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences.

We the undersigned duly appointed committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death in the United States and has been for the past 80 years. Development of novel therapeutic agents to address the large number of CVD deaths requires an in depth understanding of the structural and functional properties of human cardiomyocytes. Over the last few years we have been developing an *in vitro* paradigm to assess molecular cardiodynamics in Primary Human Cardiomyocyte in culture (PHCC). We tested the hypothesis whether endotoxemia would exhibit a marked decrease in contractile proteins and cause apoptosis in PHCC. In the current series of experiments, we induced endotoxemia using *E. coli* lipopolysaccharide (LPS) in PHCC. We investigated cell viability, induction of apoptosis and the level of contractile proteins using immunoblotting, confocal microscopy and flow cytometry. Four treatment groups with varying concentrations of LPS (0, 1, 10 and 100µg/ml) were added to wells containing one million primary human cardiomyocytes and viability was tested at 24, 48 and 72 hours post treatment using Countess automated cell counter. No significant change in the viability of PHCC was observed in both LPS and control groups. In addition, we did not find any significant alterations in the levels of TNF-alpha and Annexin V staining (a marker for early detection of apoptosis). These data indicate that the PHCC are resistant to LPS-induced induction of apoptosis and cell death as reported in other cell lines. Given that endotoxemia is associated with impairment of cardiomyocyte contraction, we hypothesized that LPS treatment would reduce the expression of contractile proteins in PHCC. Norepinephrine (NE) was used as positive control for contractile protein expression. The treatment groups included: Control; LPS, 100µg/ml; NE 10µM; LPS, 100µg/ml + NE 10µM. NE produced a significant increase in the protein levels of troponin I, tropomyosin, and myosin light chain proteins compared to the untreated control group. In contrast, LPS produced a significant decrease in troponin I, tropomyosin, and myosin light chain proteins. In addition, NE-induced increase in protein levels were significantly decreased by LPS in the LPS+NE combination treatment group compared to control groups. The fluorescence intensity of the contractile proteins was less in the LPS treated cells compared to the other treatment groups. The results gathered from confocal microscopy further strengthened immunoblot data suggesting that LPS reduced contractile protein expression. Collectively, these data suggest that LPS affect the expression of contractile proteins in PHCC without affecting the cell viability. The PHCC cell line is not sensitive to LPS-induced activation of cytokines and induction of apoptosis. It appears that LPS induced decrease in protein levels of myofibrillar and contractile proteins in PHCC might not be due to induction of apoptosis.

ACKNOWLEDGEMENTS

There have been many people that have helped me on my journey and I could not be here without their support. First, I would like to express my sincerest gratitude to Dr. Avadhesh Sharma for all of his guidance, knowledgeable scientific insight, and encouragement throughout this whole process. He has been with me every step of the way assisting and encouraging me until the task was completed. Outside the realm of science, he also shared with me his wisdom on how to become a better person and for that I am thankful. I would also like to thank my committee members, Dr. Srujana Rayalam, Dr. Richard White, and Dr. Rangaiah Shashidharamurthy for their suggestions on how to improve my project. It was a pleasure to have worked with them.

The experiments in the lab could have been done without the help of the members of the lab. I acknowledge the work Rahul Gunturi has done for this project. He has been with me for the duration of the project assisting me with each experiment above and beyond his duties. I would also like to recognize Yan Wu and Laurel Wike for their contribution to the completion of this thesis.

I would like to give much appreciation to my parents, Donnie and Arlene Collins, for their unwavering support throughout my life. They have helped made me into the person that I am today because they always helped me achieve every goal. I want to also thank my brothers, Atibah and Asibah, for always having my best interest in mind. Lastly, I want to thank the rest of my family and friends for supporting me during my journey.

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1 INTRODUCTION

1.1 Background and significance

Cardiovascular disease (CVD) is the leading cause of death in the United States and has been for the past 80 years (Greenlund, Giles, & Keenan, 2006). In 2010, a total of 595,444 people died from cardiovascular disease, which accounted for 24% of all deaths that year (Sherry L. Murphy, Xu, & Kochanek, 2012). It is estimated that about 81 million American adults are living with one or more types of CVD, with an estimated direct and indirect cost of about $503.2 billion in 2010 (Lloyd-Jones, Adams, & Brown, 2010). Many of the current therapeutics for cardiovascular disorders are designed to treat the vascular issues related to cardiovascular disease. However, the effects of these treatments on the cellular level are still relatively unknown. Development of novel therapeutics to address the large number of CVD deaths requires an in depth understanding of the structural and functional properties of human cardiomyocytes. Thus an immediate need exists for an *in vitro* paradigm to assess CVD**.**

Most of the information known about cardiovascular disease and the myocardial dysfunction has been gathered using laboratory animal models. Dogs, rabbits, mice, and rats have been the primary animals used to elucidate the structural and functional understanding of CVD. Morphological, biochemical, and electrophysiological properties of the human heart have been explained using animal models (Chlopcikova, Psotova, & Miketova, 2001). Rats have provided the most significant data in the understanding of human cardiophysiology because the rat heart

is phenotypically and physiologically similar to human fetal and pediatric heart (Chlopcikova et al., 2001). This model has been used in studies concerning contraction, ischemia, hypoxia and the toxicity of various compounds (Chlopcikova et al., 2001). There are several benefits to using animal models for cardiac research. Adult cardiac myocytes from the animal models are inexpensive to acquire; retain their viability and unique rod-shaped morphology for at least a few days (Sambrano et al., 2002). Also, these cells maintain highly organized membrane and myofibrillar structures that support contractions induced by electrical or pharmacological stimulation (Sambrano et al., 2002). Although animal models have been invaluable in establishing the knowledge about human physiology, many of the results cannot be reproduced in human models because of the physiological differences between species which leads to limited application. For those reasons, there is a need to establish an *in vitro* human model system.

1.2 Cardiac Cell Lines

Isolated embryonic and neonatal rat primary cardiomyocytes have been the most widely used models to study cardiac biology *in vitro* but their use is limited as they do not possess many adult cardiomyocyte characteristics (White, Constantin, & Claycomb, 2004). Cultured adult cardiomyocytes provide a convenient and complementary *in vitro* system with which cardiac pathology and human cardiogenesis can be investigated (Bird et al., 2003). Isolated human cardiomyocytes could help to gain insight into cardiomyopathies, particularly those associated with inter-related disorders of ion channel function, contractility and myofibrillogenesis

(Bird et al., 2003). HL-1 cell line, one of the first cardiomyocyte cell line, was derived from AT-1 cardiomyocytes which, are subcutaneous tumors obtained from the atrial cardiac muscle cells of transgenic mice (White et al., 2004). Before the discovery of the HL-1 cardiomyocyte, there were no other cells that could continuously divide, spontaneously contract, and maintain a differentiated adult cardiac phenotype through indefinite passages in culture (White et al., 2004). HL-1 has been used to study normal cardiomyocyte function with regard to signaling, electrical, metabolic, and transcriptional regulation (White et al., 2004). They also have been used to address pathological conditions such as hypoxia, apoptosis, and ischemia-reperfusion (White et al., 2004). AT-1 cells maintained a cardiomyocyte phenotype but could not be serially passaged or recover from frozen stock (White et al., 2004). The HL-1 cell line was therefore derived as it could maintain a differentiated phenotype and also be recovered from frozen stocks (White et al., 2004). While useful for studying atrial myocytes, it is limited in answering questions about ventricular cardiomyocytes and especially those pertaining to mechanisms of human cardiogenesis and cardiomyopathies (Davidson et al., 2005). Therefore, Davidson et al. used a novel, mitochondrial function-based method to immortalize primary ventricular cardiomyocyte from adult human heart tissue, and created this cell line (AC cells) by fusing SV40 transformed fibroblast heart tissue cells devoid of mitochondrial DNA with human ventricular cardiomyocyte (Davidson et al., 2005). AC cells could be passaged for over 120 generations and can be regrown from frozen stocks while retaining their original phenotype (Davidson et al., 2005). This remarkable breakthrough can serve as a useful human *in vitro* model to study cardiac

gene expression and function, during normal development and in pathological conditions at molecular levels (Davidson et al., 2005).

The primary human cardiomyocyte in culture (PHCC) used in these series of experiments were acquired from Celprogen, Inc. in San Diego California. The PHCC were acquired from donor patients and processed into single cell suspension through mechanical dissociation and enzymatic digestion. When PHCC are grown in the Celprogen growth media to 90-100% confluence, they can enter post-mitotic arrest. At this state, they exhibit characteristics most like *in vivo* cardiomyocytes.

NE is a neurotransmitter that is secreted from the adrenal medulla and is typically found in sympathetic nerve endings in the nervous system (McPhee & Ganong, 2010). NE helps body to regulate metabolism, contractility of cardiac and smooth muscle, and neurotransmission (McPhee & Ganong, 2010). ET-1 is primarily a paracrine regulator of vascular tone in the heart (Barrett, Barman, & Boitano, 2010) and a potent mitogen for vascular smooth muscle cells and cardiomyocytes (AccessPharmacy | endothelins 2012). Due to the roles NE and ET-1 play in bodily functions, both molecules were suitable candidates to promote human cardiomyocyte growth. After treatment of PHCC in NE and ET-1 for 72 hours, only NE proved to increase the amount of contractile proteins (F-actin, troponin I, tropomyosin and MLC) while ET-1 decreased their expression (Scott, 2012).

1.3 Cardiomyocyte contractile proteins

The majority of cardiomyocytes are primarily composed of contractile proteins (AccessMedicine | pathophysiologic concepts of heart failure.). The primary

contractile proteins of muscles are actin and myosin because they play a critical role in the contractile process. Actin filaments are composed of individual actin monomers that bind together (AccessMedicine | biologic tissues in orthopedics.). Myosin filaments are mainly composed of myosin heavy chain molecules (AccessMedicine | biologic tissues in orthopedics.). There are several regulatory proteins that are responsible for turning the myosin heavy chain (MHC) apparatus on and off. The regulatory contractile proteins involved in controlling the contractile apparatus and work with actin filaments to direct movement include tropomyosin, troponin-T, troponin-I, and troponin-C (AccessMedicine | biologic tissues in orthopedics.). Collectively, all these proteins compose the thin filament. Other regulatory contractile proteins are associated with the MHC and they are collectively called myosin light chains (MLCs) (AccessMedicine | biologic tissues in orthopedics).

Actin is a globular protein that performs several functions in eukaryotic cells. Actin can be found in two forms: globular and filamentous. When single monomer actin proteins called G-actin begin to align and form a linear polymer they become a microfilament called F-actin. Microfilaments are important in several cellular functions and process such as maintaining the integrity of the cytoskeleton and muscle contraction.

Tropomyosins are coiled-coil proteins that play a role in regulating the function of actin filaments during muscle contraction. Tropomyosins control the interaction of myosin to the actin filament by blocking the point of attachment when calcium levels are low. Tropomyosin accomplishes this task by working in conjunction with other regulatory protein troponin (-C,-T,-I). At low calcium levels,

troponin I (TnI) competes for position with tropomyosin which enables tropomyosin to block the myosin binding site on actin until contraction is initiated (Dominguez, 2011).

Troponin is composed of 3 regulatory proteins that work in conjunction with tropomyosin to regulate muscle contraction. The troponin complex is attached to tropomyosin and has three subunits: troponin C (TnC), troponin T (TnT), and troponin I (TnI). TnC detects calcium concentration and relieves the muscle contraction inhibition (Galinska-Rakoczy et al., 2008). TnT is a structural protein that attaches the troponin complex to tropomyosin (Galinska-Rakoczy et al., 2008). TnI is the inhibitory subunit that blocks the actin-myosin crossbridges but is relieved by TnC when calcium concentrations are high (Galinska-Rakoczy et al., 2008). Cardiac muscle cells have their own specific troponin complex. When cardiac muscle is at rest during diastole, tropomyosin covers the myosin binding site on F-actin due to low cytosolic calcium levels (Willott et al., 2010). Cardiac troponin I , which is specific to only cardiac muscles, blocks the actin-myosin interaction by inhibiting ATPase activity (Willott et al., 2010) .When calcium begins to bind to cardiac troponin C (cTnC), it induces cTnC to perform a series of conformational changes which releases the inhibitory affect cTnI (Willott et al., 2010). As a result, tropomyosin is shifted revealing the myosin binding site on the actin filament resulting in contraction (Willott et al., 2010) .

Myosin is a hexameric structure that is composed of two heavy chains, two light chains, and two regulatory light chains (AccessMedicine | biologic tissues in orthopedics.). Each MHC is composed of the same general components: a rod region,

lever arm (S_2), and a globular head (S_1) (AccessMedicine | biologic tissues in orthopedics.). The role of the rod region is to pack individual myosin heavy chains into the thick filaments (AccessMedicine | biologic tissues in orthopedics.) The globular head possess the molecular motor which is a key component in the contractile apparatus (AccessMedicine | biologic tissues in orthopedics.) . The globular head contains several domains which are responsible for converting chemical energy in the form of ATP into mechanical work and heat (AccessMedicine | biologic tissues in orthopedics.). They are the actin-binding site, the nucleotide (adenosine triphosphate [ATP])-binding site, and (3) the enzymatic (adenosine triphosphatase [ATPase]) properties (AccessMedicine | biologic tissues in orthopedics.). One essential and regulatory light chain is bound to the lever arm or S_2 region of each globular head (AccessMedicine | biologic tissues in orthopedics.). Light chains are believed to modulate the regulation of the kinetics of the crossbridge cycle (AccessMedicine | biologic tissues in orthopedics.).

MHC has two isoforms that are present in the normal human heart, α- and β-MHC (AccessMedicine | pathophysiologic concepts of heart failure.). The α-MHC predominates in the atria in a nonfailing human heart as it is more enzymatically active (AccessMedicine | pathophysiologic concepts of heart failure.). β-MHC is more present in the ventricle in a nonfailing human heart but is less active (AccessMedicine | pathophysiologic concepts of heart failure.). α-MHC is a more cardiac specific contractile protein as β-MHC can also be found in slow-twitch skeletal muscle (AccessMedicine | pathophysiologic concepts of heart failure.). Studies have shown transitions occur in the MHC isoform expression of these

chambers during various cardiomyopathies (Reiser, Portman, Ning, & Schomisch Moravec, 2001). Reiser et al. demonstrated that the expression of β-MHC predominates α–MHC in normal as well as dilated and ischemic cardiomyopathic adult human atria and ventricles (Reiser et al., 2001). This increase in β-MHC can be beneficial during the development of cardiac failure because it increases the economy of contraction by increasing stroke volume and cardiac output (Reiser et al., 2001).

The crossbridge cycle is a series of biochemical and mechanical events. The process for muscle contraction is an energy dependent mechanism. The energy used to produce contraction comes from the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) plus inorganic phosphate (P_i). During diastole, the ATPase and activity of the myosin is dormant because of chemical and physical processes in the thin filament that prevent the availability of binding sites on actin for reaction with the crossbridges (Terjung, 2010). Inhibition is relieved during systole when membrane controlled influx of calcium occurs as the calcium binds to cTnC revealing the myosin binding sites on the thin filament (Terjung, 2010). When the ATPase on the myosin head hydrolyzes ATP, this allows for the actomyosin crossbridges to be formed which results in contraction. As the calcium concentration begins to decrease in diastole and dissociate from cTnc, inhibition of the myosin binding site on actin is once again established.

Changes to the expression of contractile proteins can greatly alter the function of cardiomyocytes. Beneficial interventions such as adding NE can improve cardiac function as the expression of contractile proteins will increase. In contrast, infectious

agents such as lipopolysaccharide can cause induce sepsis in the body that could lead to myocardial depression due to the decrease in protein levels.

1.4 Sepsis-Induced Myocardial Dysfunction

With the availability of several cardiac cell lines and the increasing knowledge on the functionality of cardiac muscle contraction, the effect of sepsis on myocardial dysfunction can be better understood by using the various models. The incidence of sepsis has increase during the past 20 years making it the 10th leading cause of death in the United States (cited in Chopra & Sharma, 2007a). Approximately 500,000 to 1 million people develop sepsis each year. Sepsis is the result of the over activation of the innate immune response that is amplified far beyond the initial site of infection (Celes, Prado, & Rossi, 2012) . As a result, large amounts of pro-inflammatory mediators are produced which ultimately lead to the pathophysiology of major organs (Celes et al., 2012). Sepsis is characterized as an acute circulatory dysfunction that can have detrimental effects causing multi-organ failure (Chopra & Sharma, 2007a). When sepsis occurs, the body goes to a hyperdynamic state, with normal-to-low blood pressure and low systemic vascular resistance (Chopra & Sharma, 2007b).The mortality from septic shock syndrome ranges from 20-90% (Chopra & Sharma, 2007a). About 60% of patients admitted to the intensive care due to severe sepsis exhibit cardiac dysfunction in which the mortality for those patients range from 70-90% (Celes et al., 2012). In contrast, patients who did not show signs of myocardial dysfunction as a result of sepsis had a mortality rate of 20% (Celes et al., 2012). With such high mortality rates, sepsis is

considered the most important cause of morbidity and mortality in the ICU. The reason the high morbidity and mortality is due to the ventricular impairment of myocardial contractility which results in dysfunction (Chopra & Sharma, 2007b). Though there is an increase in cardiac output during sepsis, there is still dysfunction in the myocardium because both left and right ventricles dilate along with inotropic decrease (Levy & Deutschman, 2004). Other dysfunctions that are seen in this disease include severe depression of ejection fraction and reduced ventricular compliance (Levy & Deutschman, 2004). There is a lack of epidemiological data for myocardial dysfunction during progression of sepsis. Also, there are no suitable clinical markers for assessment of cellular myocardial function during sepsis (Sharma, 2007). For this reason, there have not been extensive studies on the cellular pathophysiology mechanism as it relates to sepsis-induced myocardial dysfunction (Chopra & Sharma, 2007a).

Studies have been performed using small mammals such as mice and rats in order to identify what pathways cause myocardial dysfunction. One of these pathways is apoptosis. There are two major apoptotic pathways have been identified. The first pathway is the extrinsic pathway activated by TNF-α receptor death domain (TRADD) (Sharma, 2007). This cell surface death domain involves tumor necrosis receptor superfamily and TNF-related apoptosis induced ligand (TRAIL) that ultimately leads to caspase-8 activation (Sharma, 2007). The second pathway is an intrinsic apoptotic pathway activated by stress-induced stimuli. This pathway is stimulated by chemical and growth factor deprivation which disturbs the function of the mitochondria which released apoptotic-inducing proteins such as cytochrome c

and Smac, and increases the expression of pro-apoptotic Bax proteins (Sharma, 2007). Once cytochrome c is release from the mitochondria, it binds with Apaf-1, ATP and caspase-9 to form an apoptosome which activates caspase-3 (Chopra & Sharma, 2007b). Increased activity of caspase-3 is mediated both by the intrinsic and extrinsic apoptosis pathways. There is also evidence that mitogen activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK) and p38-MAPK have a pro-apoptotic effect that stimulates the intrinsic pathway (Sharma, 2007). After caspase-3 activation, pro-apoptotic activating factor 2 (ATF-2) phosphorylates in the presence of the MAPK proteins and then translocates nuclear factor κB (NF-κB) from the cytosol to the nucleus (Sharma, 2007) The translocation of NF-κB causes DNA fragmentation which consequently leads to contractile dysfunction and cardiomyocyte cell death during late sepsis (Sharma, 2007).

1.5 Endotoxemia

Endotoxemia is characterized by hypotension and depression of myocardial contractility despite the fact that assessment of intrinsic cardiac function is complicated by marked increase in heart rate and alterations in preload and afterload (Gupta & Sharma, 2003). LPS-induced depressed cardiac function has been well established. *Escherichia coli* lipopolysaccharide (LPS) administration has been used to simulate the hemodynamic and inflammatory profile associated with septicemia; it is also responsible for cardiac dysfunction associated with human sepsis (Gupta & Sharma, 2003; Tissier et al., 2004) . When LPS is injected into humans at low dosage, it leads to an increase in cardiac output and vasodilation as seen in septic patients

(Levy & Deutschman, 2004). LPS can elicit a systemic inflammatory response that produces diverse cardiovascular effects in several animal models (Gupta & Sharma, 2003).

Lipopolysaccharides, also known as endotoxin, are located on the outer membrane of gram-negative bacteria and are responsible for the induction of the inflammatory response seen in infection. The immune system response is due to the release of the major pro-inflammatory cytokines which include TNF-α, IL-1β, and IL-6. Since LPS has been isolated from the plasma septic patients, it led many researchers to believe it is a contributory factor in the septic cascade (Levy & Deutschman, 2004). The LPS of all gram-negative bacteria are composed of the same general structure: a polysaccharide attached to a lipid component named lipid A (Netea, van Deuren, Kullberg, Cavaillon, & Van der Meer, 2002). Lipid A is composed of a phosphorylated β-1, 6-linked glucosamine disaccharide that is attached to long fatty acids (Netea et al., 2002). Lipid A is primarily responsible for the cytokine release upon induction of LPS (Netea et al., 2002). One gram negative bacterium typically carries about 2×10^6 LPS/lipid A molecules (Chaby, 1999). LPS molecules are recognized by LPS-binding proteins (LBP) once in contact with the host cell. The LPS-LBP complex is then transported to membrane bound receptor protein named CD14, which is a glycoprotein imbedded to the membrane of myeloid cells by glycosly-phosphatidlyl-inositol (GPI) anchor (Chaby, 1999). CD14 also works in conjunction with another protein, a class of receptors called Toll-like receptors (TLRs)(Netea et al., 2002).TLRs are transmembrane proteins that have a conserved leucine rich motifs which are common in other recognition proteins of the

innate immune system (Heumann & Roger, 2002). There are 10 members in the TLR family (TLR1-TLR10) but TLR4 is the likely candidate to transmit the LPS signal to the cytoplasm in mammals (Chaby, 1999; Heumann & Roger, 2002). Shimazu et al. (1999) has also discovered that along with TLRs, a molecule named MD-2 is associated with TLR-4 to assist in the responsiveness of the cell to LPS (Shimazu et al., 1999). Once the cytokines are released from LPS stimulated cells, they stimulated different metabolic, hormonal, and neuroendocrine changes in cells of different tissues and organs (Chaby, 1999). The endotoxin shock cascade can ultimately lead to physiological effects on the body which include fever, tissue damage, vascular dysfunction, myocardial depression and death.

In addition, Sharma and coworkers (Chopra & Sharma, 2007b) observed the expression of caspase-3 increase in adult rat ventricular myocytes (ARVM) when they were inoculated with LPS. Also, it was determined that LPS caused the cleavage of contractile proteins which adversely affected ARVM function. Therefore, we wanted to reproduce the findings that were seen in the animal model in PHCC because the results will have better translation to what happens to human cardiomyoctes *in vivo*.

2 SPECIFIC AIMS AND OBJECTIVES

With the incidence of sepsis steadily increasing in the population, it is important to research the many functions that are being compromised during each episode. Having a better structural and functional understanding of the contractile process in cardiac cells during sepsis-induced myocardial dysfunction can help physicians combat the disorder with greater efficiency. Much of our understating about the mechanisms behind over activation of the immune system and how it affects the heart has been gathered using animal models. Though these models have provided a great benefit to our understanding, there are still physiological and morphological differences that decrease the amount of data that can be translated into human models. As a result, PHCC can be an established cell line that can increase our knowledge on human cardiomyocyte pathophysiology during sepsis.

In the current series of experiments, we will produce endotoxemia in primary human cardiomyocytes in culture and examine the level of contractile proteins and viability. We hypothesize primary human cardiomyocytes treated with LPS will show a marked decrease in contractile proteins and exhibit apoptosis. To address this hypothesis, the following specific aims were developed:

Specific Aim #1: To determine the effect of varied doses of LPS on the viability and apoptosis of PHCC.

Specific Aim #2: To determine the effect of NE on LPS-treated PHCC on the levels of contractile and myofibrillar proteins.

3 MATERIALS AND METHODS

3.1 PHCC Cell Culture

Primary Human Cardiomyocyte Cultures (PHCC), which was procured from adult cardiac tissue donors, was purchased from Celprogen, San Pedro, CA. Upon arrival of the frozen vial, cells were completely thawed and transferred into 9 mL of Celprogen's Human Cardiomyocyte Cell Culture Complete Growth Media with serum provided by Celprogen. After centrifugation, cells were reconstituted in 7 mL of growth media and plated on a T-25 extracellular matrix (ECM) pre-coated flask (Celprogen, San Pedro, CA). Cells were incubated at 37°C, 5% CO_2 in a humidified incubator. During continued growth, the medium was changed routinely every 24 hours.

When attached cells reached 60-70% confluence, they were trypsinized with 3 mL of Trypsin EDTA (Celprogen, San Pedro, CA) and neutralized with 7 mL of Complete Growth Media. At the end of each passage, some cells were reconstituted in growth media and plated in an ECM pre-coated T-75 flask for further growth. The remaining cells were reconstituted in freezing media, transferred into cryogenic vials, and placed into a liquid nitrogen tank vapor phase.

3.2 Preparation for LPS-EB

To prepare LPS-EB (lipopolysaccharide from E. coli 0111:B4) stock solution, 5mg LPS-EB was received from InvivoGen (San Diego, Ca). The sterile stock

solution was prepared by adding 1ml of sterile water to the LPS-EB and then homogenized creating a 5mg/mL solution. In order to make a working solution of LPS-EB of 1mg/mL, 100µL of the stock solution was added to 400µL of sterile water.

LPS concentrations of 1, 10, and 100µg/mL were also used for the viability, immunocytochemistry, and western blot experiments. To prepare the 100µg/mL solution, 300µL will be taken from the stock 1mg/mL solution of LPS and added to 700µL of growth media (Celprogen). This now 300 µg/mL solution will be diluted to 100µg/mL when 100µL of this solution is added to its respected well making a total volume of 3mL. To make the 10µg/mL solution, 100µL will be taken from the previous 300µg/mL solution and be added to 900mLs of growth media. Again, this creates 30µg/mL solution and when 100µL is added to appropriate well, it will create a final concentration of 10µg/mL in a 3mL solution. For the final concentration, 100µL will be taken from the 30µg/mL solution and be combined with 900mL of growth media. 100µL of this solution (3µg/mL) will be added to the well designated for 1µg/mL concentration of LPS-EB.

3.3 Preparation of 10mM NE Stock Solution

To make the NE (10mM) stock solution, 1.92mg of L-ascorbic acid 2-phosphate sesquimagnesium salt (Sigma-Aldrich) was dissolved in 25mL of cell culture grade water (Sigma-Aldrich). This was a 0.3mM L-ascorbic acid solution. In a chemical safety fume hood, 33.73mg norepinephrine bitartrate salt monohydrate

(Sigma-Aldrich) was added to 10mL of the 0.3mM L-ascorbic acid solution. This was a 10mM norepinephrine stock solution. The solution was then sterile filtered with a sterile filter syringe. The stock solution was made into working solution aliquots and stored at -20°C.

3.4 Treatment of PHCC with LPS-EB

After inspection, one-T75 Flask 80% confluence of PHCC was removed from incubation. The growth media in the flask was discarded and 5mL of Trypsin EDTA was added to the T75 flask for 3 minutes. After the allotted time, 10mL of PHCC growth media was added to neutralize the reaction. The contents of the flask were then transferred into a sterile 15mL conical tube. The tube was centrifuged at 100G for 7 minutes. The supernatant was discarded leaving a pellet at the bottom of the tube. The pellet was reconstituted with 10mL of PHCC growth media. A sample from the tube was taken to be counted using the Trypan Blue and hemocytometer method. Next, 4 wells in a 6-well plate were labeled: control, 1µg/mL LPS, 10µg/mL LPS, and 100µg/mL LPS. Calculations were performed to measure how many milliliters from the PHCC solutions would constitute 1 x 10^6 cells. The appropriate amount of milliliters were removed from the PHCC solution and added to each of the 4 wells so that each well had approximately 1 x 10^6 cells. PHCC growth media was added to each well so that the total volume per well equaled 2.9mL. The wells were counted again as to have a more accurate number for the amount of cells in each well. Then 100µL of 1µg/mL, 10µg/mL and 100µg/mL of LPS was added to each well. The plate

was incubated in 37°C, 5% CO_2 in a humidified incubator. At various time points (24 hours, 48 hours, 72 hours) 200µL of supernatant were removed from each well and stored in -70°C freezer. Another cell count (live and dead cells) was taken at each time point and recorded. After 72 hours, the cell pellet from each well was harvested.

3.5 Cell Growth with LPS and NE

PHCC were grown in the presence of LPS and NE to measure viability and protein levels. After inspection, PHCC were removed from a T-75 flask once the cells reached 80% confluence. The supernatant was discarded and 5mL of Trypsin EDTA was added to the flask for 3 minutes. After 3 minutes, 10mL of growth media was added to neutralize the solution. The cells were resuspended and added to a 15mL conical tube and centrifuged at 100G for 7 minutes. The supernatant was then discarded and 10mL of growth media was added to the pellet. After the PHCC were resuspended, they were counted using Countess® Automated Cell Counter. Calculations were performed to add approximately 1 million cells to 4 wells for each treatment group (LPS100µg/mL, NE 10µM, LPS100µg/mL + NE 10µM, and control). This was performed in triplicate to insure accuracy of the results. Growth media was added to each well so the total volume came to 3mL. For the LPS 100µg/mL group, 30µL was taken from the stock LPS (1mg/mL) and added to respective wells to create 100µg/mL of LPS in the solution. To create 10µM treatment group, 3µL was taken from the stock NE (10mM) and added to appropriate wells. The viability was measured daily for 72 hours and the pellets were collected at

the end. Some PHCC was used for immunoblot analysis and the other was used for confocal analysis.

3.6 Determination of Number of Cells

After each designated time point, the PHCC were detached from the well by slowly pipetting several times. A small sample was taken from each treatment group and Trypan blue was used to measure the viability of the cells using a Countess® Automated Cell Counter (Life Sciences). The number of live and dead cells was counted two times, with cell viability being calculated for each count.

3.7 Enzyme Immunoassay

For the Enzyme Immunoassay experiment, 10, 100, and 1000ng/mL of LPS were used to measure the dose response of TNF-alpha of PHCC in the presence of LPS. To create these concentrations, 15uL of 1mg/mL of LPS working solution was placed in a 15mL conical tube. 485µL of PHCC growth media (Celprogen San Diego, Ca) was added to the tube to make 500µL of 1000ng/mL LPS. Next, 50µL of 1000ng/mL solution was placed in another 15mL conical tube. Then, 450µL of PHCC growth media was added into the tube creating 500µL of 100ng/mL solution. To create the 10ng/mL LPS solution, 50µL of the 100ng/mL LPS solution was removed and placed in an empty 15mL conical tube along with 450µL of PHCC growth media. 100µL of each LPS concentration was added to each well containing approximately 1 million PHCC in 2.9mL of growth media. The cells were then incubated in 37°C and 200µL was collected from each group at various time points

(30 minutes, 2 hours, 6 hours, and 24 hours) and stored in -80°C freezer. To measure the TNF-alpha levels for the samples collected, TNF-alpha (human) EIA kit was used (Enzo Life Sciences).

3.8 Flow Cytometry

The PE Annexin V Apoptosis Detection Kit I was used to obtain the flow cytometry data. PHCC were grown in the presence of LPS (1, 10, 100 µg/mL) for 24 hours. After the 24 hour time point, each PHCC treatment group was harvested and washed twice with cold PBS and then resuspend in 1X Binding Buffer at a concentration of 1 x 10^6 cells/mL. 100µL of each cell solution was then transferred to a 2mL eppendorf tube. Next, 5µL of PE Annexin V and 5µL of 7-AAD was added to each tube. The PHCC were gently vortex and incubate for 15 min at RT (25°C) in the dark. Lastly, 400µL of 1X Binding Buffer was added to each tube and then analyzed by flow cytometry within 1hr using Accuri C6 Flow Cytometer (BD Biosciences).

3.9 Immunocytochemistry

After 24 hours of growth on the laminin-coated coverslip, the media was removed from the coverslip and rinsed three times with PBS. Five hundred µL of 0.8% paraformaldehyde in PBS was added to the attached cells for 30 minutes at 30°C. The coverslip was then rinsed three times with phosphate-buffered saline

(PBS) for 2 minutes. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 3 minutes at room temperature. Upon removal of the permeabilization buffer, the coverslips were washed three times with PBS then treated with blocking solution containing 2% BSA in PBS for 30 minutes at room temperature. Rabbit monoclonal antibody for troponin (Abcam) and mouse monoclonal antibody for tropomyosin (Invitrogen) was pipette onto the coverslip in a 1:200 and 1:50 dilution, respectively for 16 hours. Following three washes with PBS, the secondary dyes, Alexa Fluor 532 (Molecular Probes) and Alexa Fluor 488 (Molecular Probes), were added for a 1 hour incubation to fluoresce troponin and tropomyosin, respectively. F-actin was stained with Alexa Fluor 568 phalloidin for a 20-minute incubation period. Following another three washes with PBS, the nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI) *FluoroPure™ grade (Molecular Probes) for 3 minutes. A small drop of glycerol was added to the microscopy slide. The coverslip was carefully placed onto the slide and secured with clear nail polish.

3.10 Confocal Imaging

Winship Cancer Institute of Emory University Cell Imaging and Microscopy Core provided usage of their Zeiss LSM 510 META confocal microscope. Argon, Helium Neon (HeNe), Cyanine3 (Cy3), and Blue Diode were the laser lines utilized for imaging the cardiomyocytes. The settings (laser power, filters, dichroic mirrors, polarization voltage, and scan speed) were optimized using the brightest stained cells, which were the untreated cells. The settings were kept constant at 40x magnification

for all samples thereafter to ensure that valid comparisons could be made between measurements from different images. Five image areas were taken for each treatment group.

Confocal images were analyzed using Image J 1.45s software developed at the National Institutes of Health (NIH). The area of each cell was outlined in the differential interference contrast (DIC) channel with the freehand selection tool. The fluorescent intensity of F-actin, troponin, and tropomyosin were all measured for each cell at the same cell area. The area and fluorescent intensity of the nucleus was measured by outlining the nucleus in the DAPI channel with the freehand selection tool for each individual cell. The cell number, cell area and intensity, and nuclear area and intensity were measured and collected for statistical analysis.

3.11 Western Blot

Cells were collected and lysed with 1% Triton X-100 containing a protease inhibitor cocktail. Lysates were homogenized and centrifuged at 100G for 7 minutes and the resulting supernatant was collected. Protein samples were quantified using Pierce Micro BCA Protein Assay Kit (Thermo Scientific, USA). Proteins were separated by SDS-Page electrophoresis using Ready Gel 4-15% Tris-HCl Gels (Bio-Rad, USA). Each lane contained 10μg of proteins. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). Rabbit monoclonal cardiac troponin I antibody (Abcam) was visualized with bovine anti-rabbit IgG-HRP secondary antibody (Santa Cruz). Mouse monoclonal Myosin Light Chain (MYL3)

antibody (Santa Cruz) was visualized with goat anti-mouse IgG-HRP secondary antibody (Santa Cruz). Goat polyclonal actin antibody and goat polyclonal tropomyosin antibody were visualized with bovine anti-goat IgG-HRP secondary antibody (Santa Cruz). All blots were visualized using the A/T2000 XR Automatic Film Processor (Air Techniques Inc). Western blot data was analyzed with UN-SCAN-IT Gel 6.1.

3.12 Statistical Analysis

The data obtained from the cell counting protocols, protein expression studies and confocal fluorescence measurement were assessed using Analysis of Variance (ANOVA). Once a significant F value is obtained, a *post hoc* Students Newman Keuls test was employed to assess inter group (time-dependent and/or dose dependent) significant difference among various groups. The criterion for significance was $p \leq 0.05$.

4 RESULTS

4.1 Growth Pattern of PHCC

PHCC displayed various morphologies ranging from circular to polygonal upon visual inspection in the flask. As the PHCC began to grow in extracellular matrix coated flask, they began to form a syncytium with other nearby cardiomyocytes. PHCC took approximately 72 hours to reach 80% confluence in a T-25 flask (Figure 1). At 80% confluence, the number of cells that were harvested ranged between 3-4 x10^6.

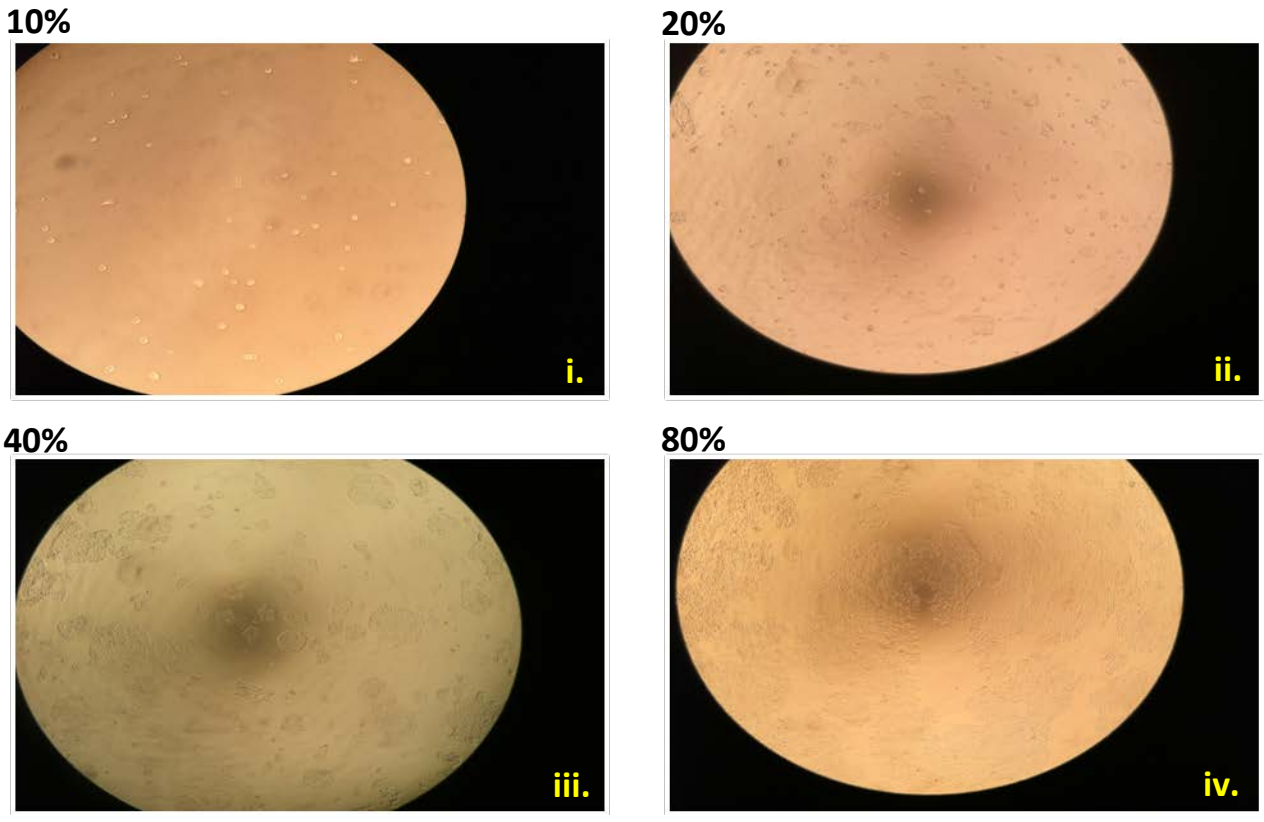


Figure 1: Representative pictographs of PHCC over a 72-hour time period at 40X magnification. (i) 0 hour (ii) 24-hours (iii) 48-hours (iv) 72-hours.

4.2 The Effect of LPS-EB on the Viability of PHCC

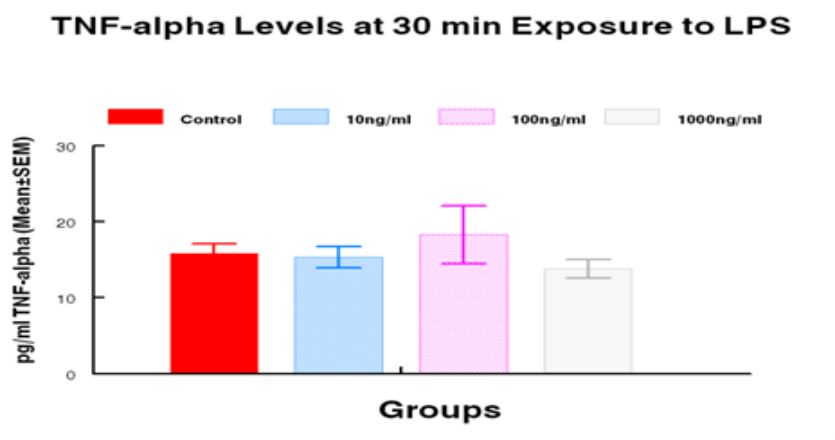
The Trypan blue counting method and the Countess® Automated Cell Counter were used to measure the effect of LPS-EB on the percent viability of the PHCC following treatment. To ensure the accuracy, the PHCC in each well were counted in duplicate. The viability remained constant as LPS-EB demonstrated it had no significant effect on the viability of the PHCC. The viability between treatment groups and time points ranged between 65%-87% (Table 1) and showed no statistical significance. Apparently with the treatment of LPS the viability of the PHCC increased as time progressed. The PHCC viability began to increase suggesting they began to recover from the introduction of the endotoxin.

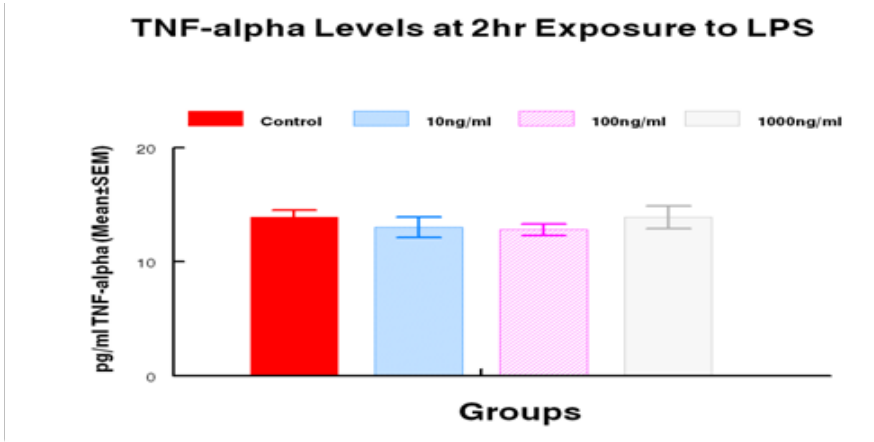
Table 1: The Effect of LPS (1, 10, 100µg/ml) on the viability of PHCC at 72 hours post treatment

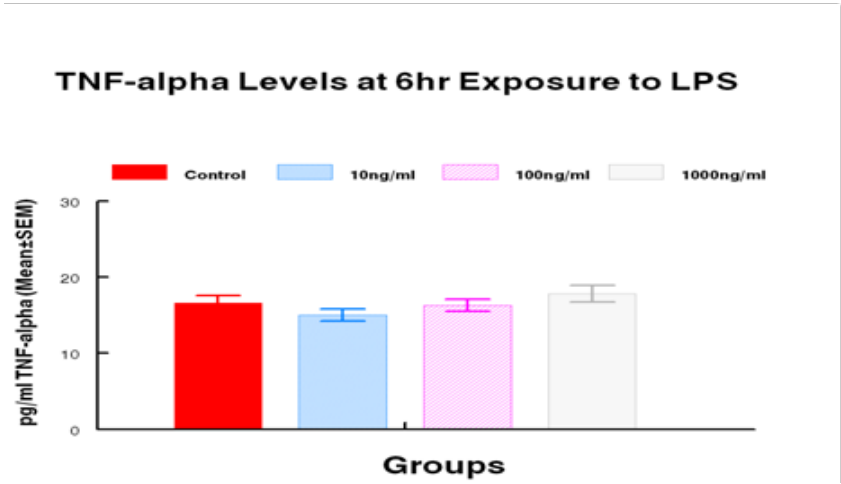
	Mean Viability (%) ± SEM			
Treatments	**0 hours**	**24 hours**	**48 hours**	**72 hours**
Control	65 ± 4	75 ± 11	70 ± 2	87 ± 1
1ug/ml LPS	63 ± 4	75 ± 2	71 ± 10	84 ± 2
10ug/ml LPS	67 ± 2	72 ± 2	74 ± 2	86 ± 2
100ug/ml LPS	76 ±10	73 ± 2	79 ± 2	87 ± 1

4.3 The Effect of LPS-EB on TNF-alpha Levels

TNF-alpha (human), EIA kit from Enzo Life Sciences was used to measure the levels of TNF-alpha in PHCC after LPS-EB treatment. Three concentrations of LPS-EB (10,100, 1000ng/mL) were used in treatment. These concentrations of LPS were used on the basis of preliminary data and reports from literature. No significant changes in the TNF-alpha levels were observed at any time point (30 minutes, 2 hours, 6 hours, and 24 hours) or concentrations.







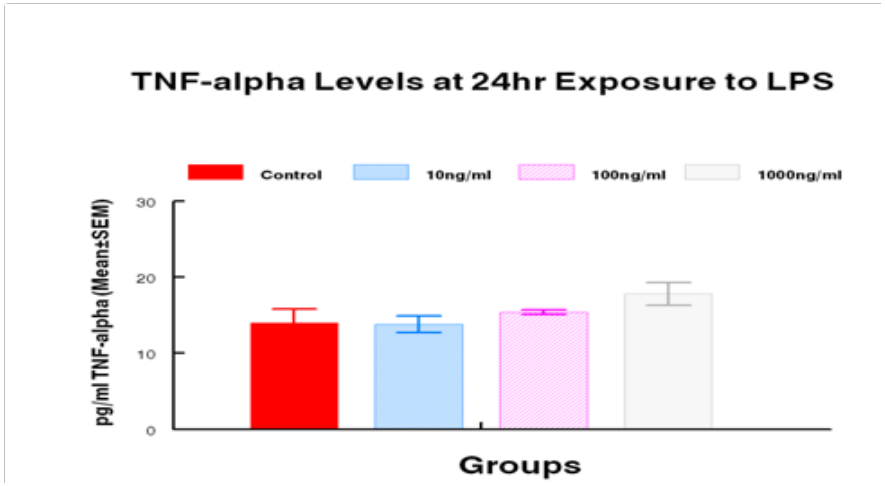


Figure 2: TNF-alpha levels measured in pg/ml for various doses of LPS (10, 100, 1000ng/mL) at several time points (30min, 2hr, 6hr, and 24hr). There was no observed significant difference in TNF-alpha levels between groups at any time point.

4.4 Effect of LPS-EB on Apoptosis of PHCC

Annexin V : PE Apoptosis Detection Kit I from BD Biosciences was used to measure apoptosis of the PHCC at various doses of LPS (1, 10, and 100µg/mL). After being exposed to the various doses of LPS for 24 hours, the PHCC exhibited no evidence of significant apoptosis occurring between each groups.

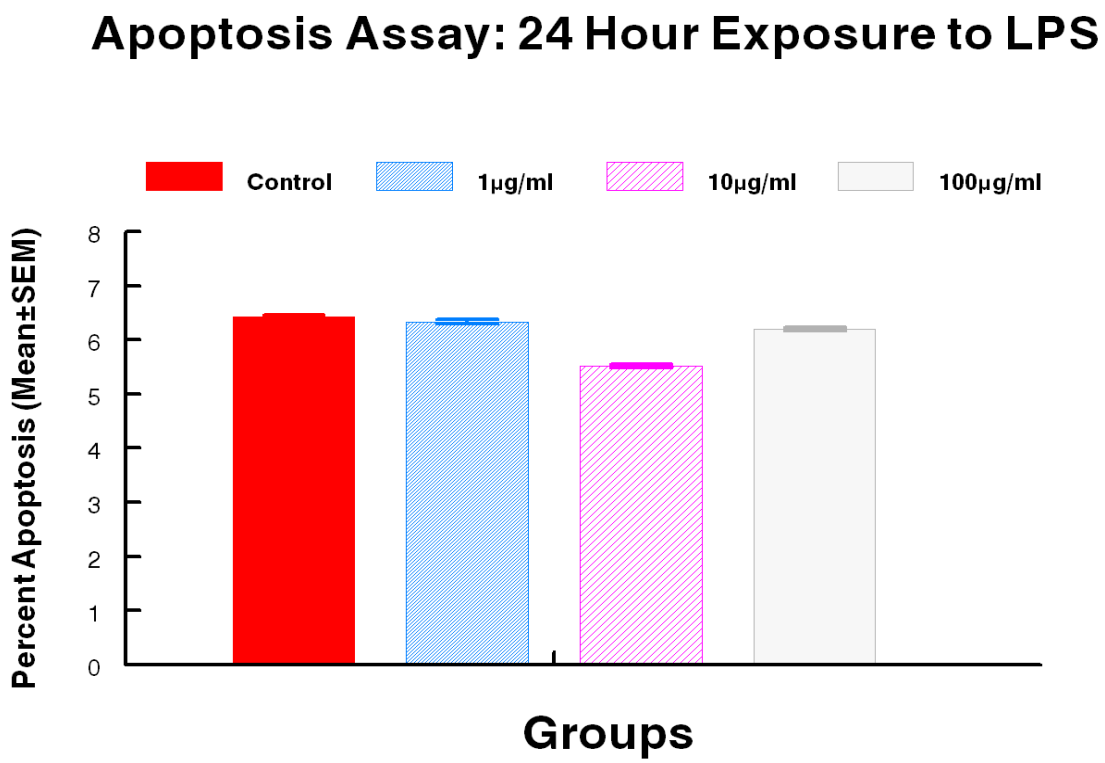


Figure 3: The effects of LPS (1, 10, and 100µg/mL) on apoptosis in PHCC. There was no significant increase in apoptosis in any treatment group suggesting PHCC is resistant to LPS-induced induction of apoptosis and cell death. Flow cytometry was performed on Accuri C6 Flow Cytometer using Annexin V: PE Apoptosis Detection Kit-BD Biosciences.

4.5 The Effect of NE on LPS-treated PHCC on the Viability

Trypan blue counting method and Countess® Automated Cell Counter were used to measure the viability of LPS-treated PHCC when in the presence of NE. Trypan blue counting method and hemocytometer were used to calculate the viability of PHCC in NE after a 72 hour period. In Table 2, the viability remained stable in NE (1, 10, 100µmol) treated cells. The percent viability varied between 69-76%. The changes between treatment groups were not statistically significant. The PHCC were counted in triplicates to ensure accuracy. In Table 3, LPS-treated PHCC were grown in 10µmol NE and exhibited relatively stable viability data. The viability ranged from 56-75% over a 72 hour period. The results were not significant. The assay was performed in triplicate and counted twice to ensure accuracy of the data.

Table 2: Effect of NE (1, 10, 100µmol) on the viability of PHCC at 72 hours post treatment

Treatment	**Mean Viability (%) ± SEM**
Control	76 ± 2
NE (1 µmol)	74 ± 2
NE (10 µmol)	64 ± 15
NE (100 µmol)	69 ± 10

Table 3: Effect of LPS (100µg/mL) in the presence of NE (10µmol) on the viability of PHCC at 72 hours post treatment.

Treatment	0 hours	24 hours	48 hours	72 hours
Control	65 ± 4	75 ± 11	70 ± 2	87 ± 1
LPS + NE	56 ± 2	75 ± 4	72 ± 0.3	58 ± 0.6

4.6 Confocal Microscopy

Confocal microscopy was used to analyze the effects of NE on LPS-treated PHCC on the expression of the contractile proteins F-actin, troponin I, tropomyosin and the nucleus through detection of fluorescent staining.

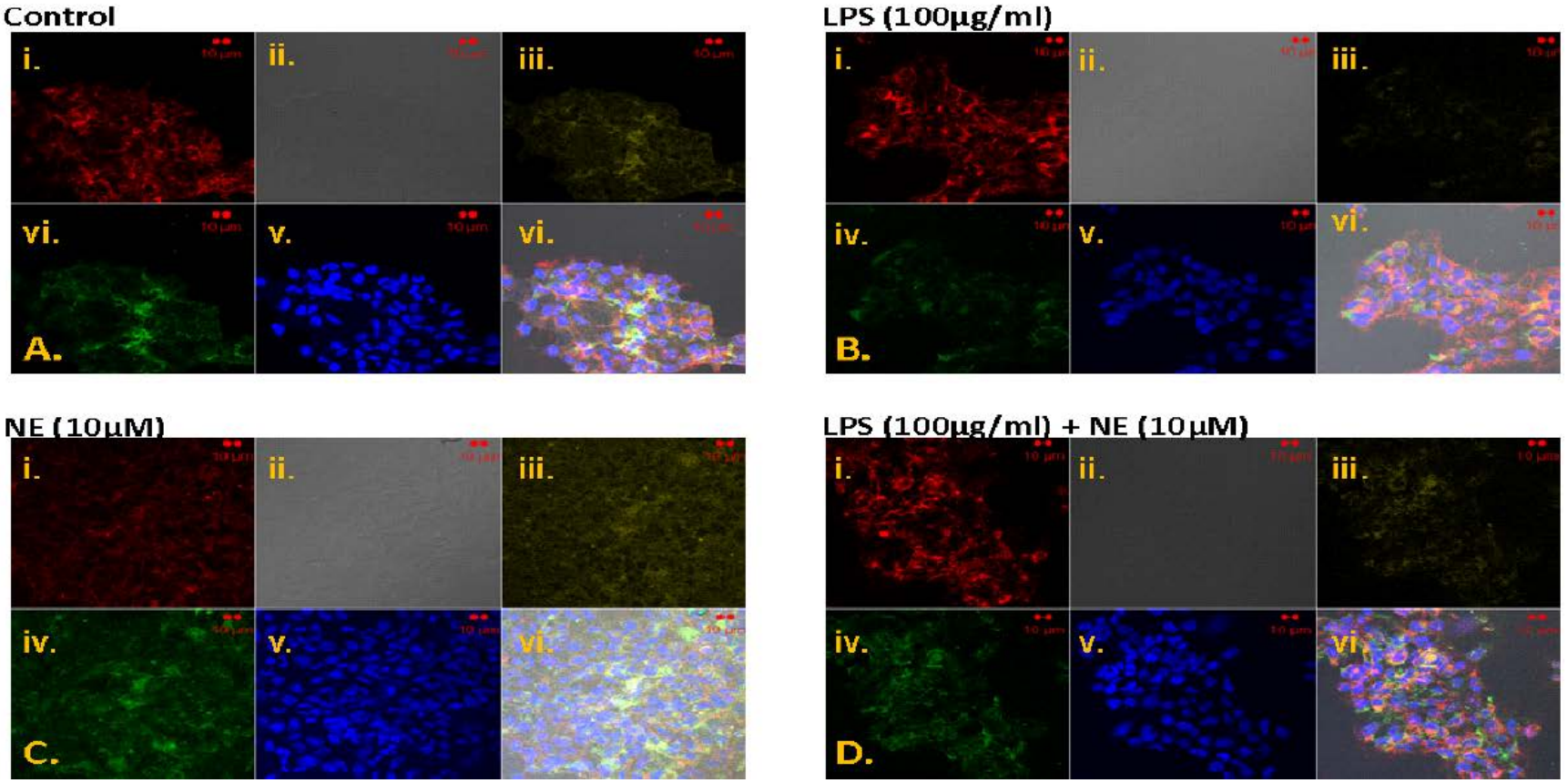


Figure 4: The effects of LPS (100µg/ml) and NE (10 µM) on the expression of contractile proteins F-actin (i), troponin I (iii), tropomyosin (iv) & the nucleus (v). PHCC were immunostained with monoclonal antibodies and detected with Alexa Fluor Phalloidin 568 (i), Alexa Fluor 532 (iii), Alexa Fluor 488 (iv), and DAPI (v). Representative images from DIC (ii) and overlay (vi) are presented. The most significant decrease of F-actin troponin I, tropomyosin, and DAPI fluorescence/ cell area intensity was observed in the LPS treatment group (B. i-vi). Confocal microscopy was performed on Zeiss LSM 510 META microscope.

4.7 Confocal Analysis of F-actin Fluorescence

Quantitative analysis of F-actin fluorescence per cell was achieved using Image J software and SPSS. There was a statistically significant decrease ($p \leq .05$) in the amount of fluorescence of F-actin in the LPS-treated PHCC as compared to control group. LPS caused a 33% decrease in the expression of F-actin. NE appeared to have provided a protective effect when placed in LPS-treated cells as the decrease was only limited to 5%. NE alone also decreased the amount of F-actin by 30%.

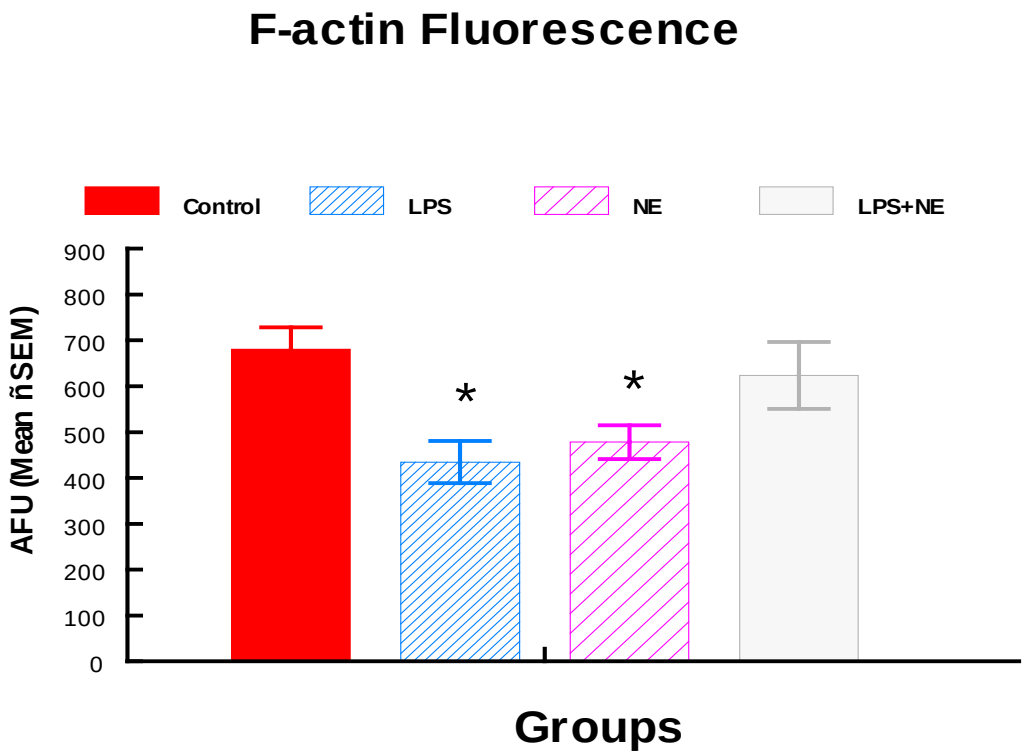


Figure 5: Quantitative analysis of F-actin protein fluorescence. After treatment of each group (LPS 100µg/ml, NE 10µmol, LPS 100µg/ml+ NE 10µmol) for 24 hours, PHCC were immunostained to F-actin with Alexa Fluor Phalloidin 568 conjugate. AFU, Arbitrary fluorescence unit. Number of cells analyzed: 44 (control); 18 (LPS); 26 (NE); 20 (LPS+NE) ($p \leq .05$)

4.8 Confocal Analysis of Tropinin I Fluorescence

Quantitative anaylsis to measure the fluorescence of troponin I fluorescence was done by Image J and SPSS. There was 75% decrease in the fluorescence of tropinin I in PHCC treated with LPS. PHCC treated with NE only showed a 50% decrease in fluoresence but provided a protective affect when NE was added to LPS-treated PHCC.

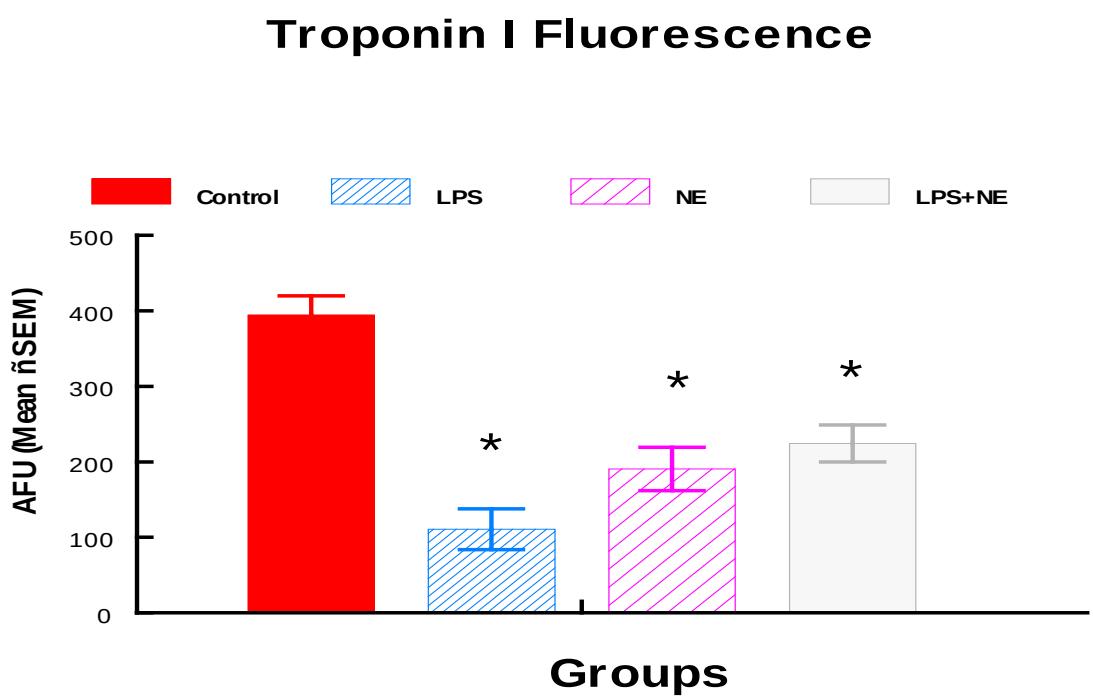


Figure 6: Quantitative analysis of troponin I protein fluorescence. After treatment of each group (LPS 100µg/ml, NE 10µmol, LPS 100µg/ml+ NE 10µmol) for 24 hours, PHCC were immunostained to troponin I with monoclonal anti-cardiac troponin I primary antibody and detected with Alexa Fluor 532. AFU, Arbitrary fluorescence unit. Number of cells analyzed: 44 (control); 18 (LPS); 26 (NE); 20 (LPS+NE)

4.9 Confocal Analysis of Tropomyosin Fluorescence

Quantitative analysis of tropomyosin fluorescence revealed a 50% decrease in PHCC treated with LPS alone when compared to control. NE appeared to have ameliorated the decrease in protein fluorescence when added to LPS-treated PHCC limiting the decrease in fluoresces to only 20%.

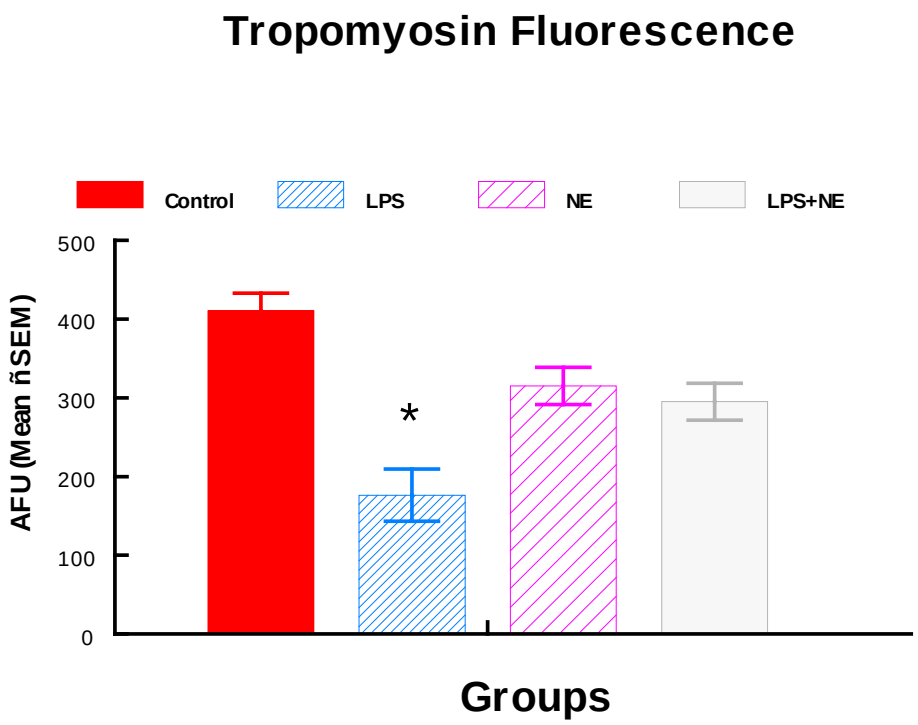


Figure 7: Quantitative analysis of tropomyosin protein fluorescence. After treatment of each group (LPS 100µg/ml, NE 10µmol, LPS 100µg/ml+ NE 10µmol) for 24 hours, PHCC were immunostained to tropomyosin with monoclonal tropomyosin primary antibody and detected with Alexa Fluor 488. AFU, Arbitrary fluorescence unit. Number of cells analyzed: 44 (control); 18 (LPS); 26 (NE); 20 (LPS+NE)

4.10 Confocal Analysis of Nuclear Fluorescence

Quantitative changes of nucleic fluorescence of 4' 6-diamidino-2-phenylindole (DAPI) were measured in the LPS-treated, NE-treated, and LPS/NE-treated PHCC. Nuclear fluorescence had a 40% decrease in PHCC treated with LPS. No significant changes are seen to the nuclear fluorescence in NE-treated cells when compared to the control. NE significantly counteracted the LPS-induced decrease of nuclear fluorescence in PHCC with only a 15% decrease.

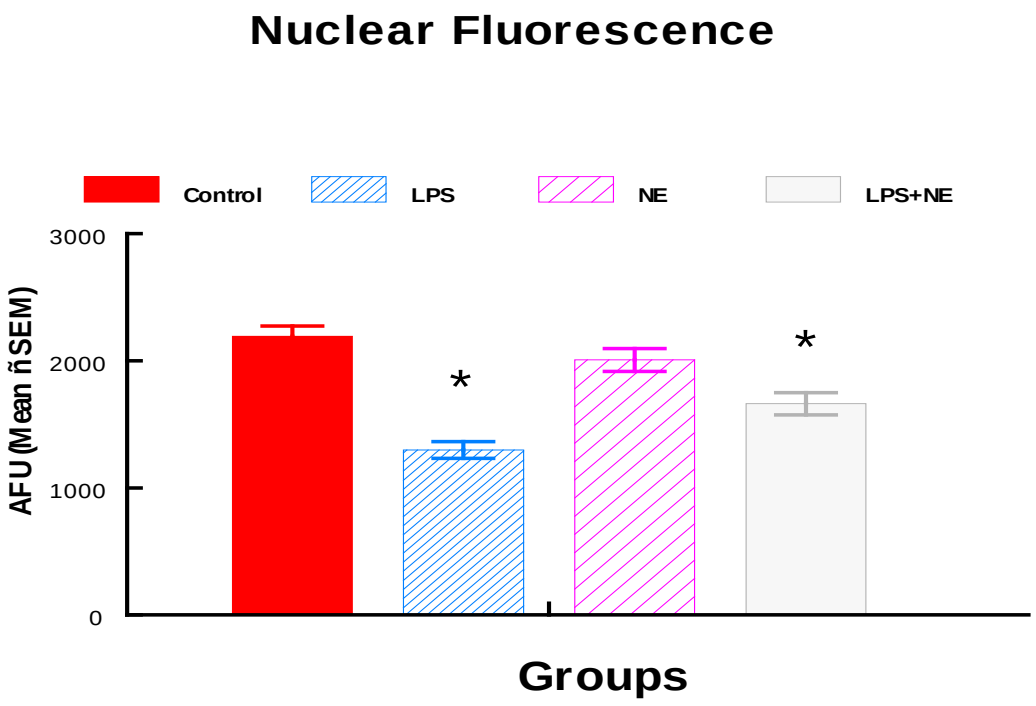


Figure 8: Quantitative analysis of changes in nucleic fluorescence of 4′, 6-diamidino-2-phenylindole (DAPI). After treatment of each group (LPS 100µg/ml, NE 10µmol, LPS 100µg/ml+ NE 10µmol) for 24 hours, PHCC were immunostained with 300µM of DAPI. The fluorescence intensities were determined for each treatment group under the same conditions. AFU, Arbitrary fluorescence unit. Number of cells analyzed: 44 (control); 18 (LPS); 26 (NE); 20

4.11 Immunoblot Analysis

Immunoblot analysis revealed significant changes in protein expression of tropomyosin and MLC of LPS-treated PHCC. For these two proteins, the levels of expression were significantly decreased (50% and 45%, respectively) when compared to the control. In contrast, troponin I protein levels were not significantly altered in LPS-treated groups. For tropomyosin and MLC, NE ameliorated the LPS-induced decrease of protein expression.

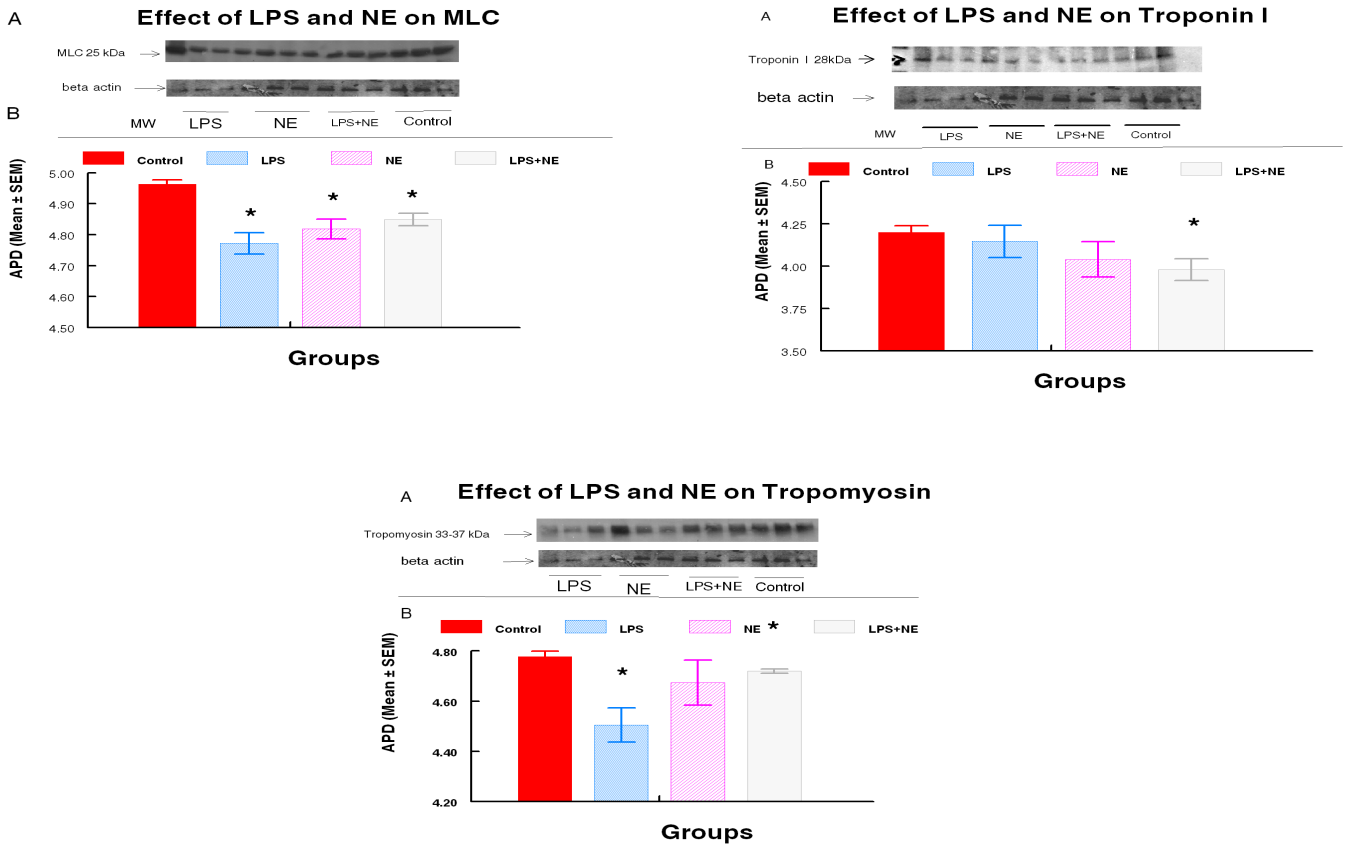


Figure 9 Effect of LPS and (NE) on contractile protein expression. Western blot analyses (10µg of protein per lane) were performed to determine protein expression of myosin light chain (MLC-1), tropomyosin, and cardiac troponin I on LPS-treated PHCC in NE. Protein expression for all three proteins were decrease in the presence of LPS.NE appeared to ameliorate the LPS-induced decrease when added to solution. (A) Represents the protein levels in each protein in treatment groups. (B) Represent the average pixel density (APD) for each treatment group.

5 DISCUSSION

Endotoxemia is characterized by hypotension and depression of myocardial contractility despite the fact that assessment of intrinsic cardiac function is complicated by marked increase in heart rate and alterations in preload and afterload (Gupta & Sharma, 2003). LPS-induced depressed cardiac function has been well established as it can elicit a systemic inflammatory response that produces diverse cardiovascular effects in several animal models (Gupta & Sharma, 2003). Though these models have provided a great benefit to our understanding, there are still physiological and morphological differences that decrease the amount of data that can be translated into human models. PHCC is an established cell line that can increase our knowledge on human cardiomyocyte pathophysiology during endotoxemia. We produced endotoxemia in primary human cardiomyocytes in culture and examined the level of viability. PHCC grew in the presence of LPS (1, 10, 100µg/mL) for 72 hour and viability of the cells were determined every 24 hours. PHCC were resistant to LPS-induced cell death as they were unaffected by increasing dose and length of exposure to LPS. One reason for the lack of response could be due to TLR4 receptors not being fully developed. Taverner et. al. demonstrated that TLR4 molecules are critical for the LPS-induced myocyte dysfunction in mice (Tavener et al., 2004). Without TLR4 receptors, the mice did not recognize that they were in an endotoxic environment which in turn did not elicit an immunological response. The PHCC did not show typical cardiomyocyte morphology as some cells were circular and pentagonal in shape. For this reason, PHCC may not have been fully differentiated which could lead to TLR4 receptors not being fully expressed. As a result, increasing

doses of LPS did not produce large amounts of cell death. An immune response was not seen during the enzyme immunoassay experiments with LPS (10, 100, 1000ng/mL) treated PHCC as the levels of TNF-alpha, one of the first steps in the apoptosis cascade, were not increased. An explanation for the lack of significant TNF-alpha levels could be due to the fact that PHCC are not as responsive to LPS as other cell lines. Studies performed by Kojima et al. (2003) using human monocytic THP-1 cells were treated with 1µg/mL LPS from *E. coli* and exhibited a 9000pg/mL increase in TNF-alpha levels (Kojima et al., 2003). In contrast, PHCC treated with the LPS 1000µg/mL only exhibited 16pg/mL increase in TNF-alpha levels.

Norepinephrine regulates smooth and cardiac muscle contraction as well as other metabolic processes (McPhee & Ganong, 2010). In our laboratory, we demonstrated that NE increased the expression of contractile proteins without affecting the viability (Scott, 2012). In the current study, treatment with varied dose of LPS produced no changes to the viability of the PHCC in presence and absence of NE at any time point studied.

Apoptosis is a naturally occurring mechanism that mediates programmed cell death of physiological and pathological processes (Arends, Morris, & Wyllie, 1990), and is characterized by morphological changes to the nucleus and cytoplasm that lead to the degradation of chromatin and ultimately cell death. An apoptotic assay was conducted to determine if LPS would cause an increase in apoptosis in PHCC. No significant occurrence of apoptosis was observed at any concentration of LPS (1, 10, 100 µg/ml) as the amount of cell death detected by the Annexin V ranged between 5-7%. Having a low percentage of apoptotic cells is not sufficient enough to

demonstrate that the PHCC are affected by LPS treatment. Further studies are warranted to confirm these findings.

The majority of cardiomyocytes are primarily composed of contractile proteins (AccessMedicine | pathophysiologic concepts of heart failure.). The primary contractile proteins of muscles are actin and myosin because they play a critical role in the contractile process. Experiments were performed to determine whether LPS affected the contractile machinery of PHCC. Immunocytochemistry was used to analyze if LPS affected the levels of F-actin, troponin I, tropomyosin, and the nucleus. Also, we wanted to determine whether LPS-treated PHCC with added NE would have any beneficial effect in regards to protein fluorescence. After 24 hours of exposure to LPS, all proteins and the nucleus had a decrease in fluorescence suggesting LPS adversely affect the amount of expression of the contractile proteins and the nucleus. Troponin I had the largest decrease in fluorescence relative to the control which suggested that it was most adversely affected from the LPS treatment. Although LPS did not affect cell viability it did however alter the amount of expression of contractile proteins. Similar results were seen in immunoblot analysis of protein levels when exposed to LPS and NE for 24 hours. The protein levels that were measured included troponin I, tropomyosin, and myosin light chain 1 (MLC1). Tropomyosin and MLC1 showed a decrease in protein levels when PHCC was exposed to LPS for 24 hours. The LPS-induced decrease was lessened in both tropomyosin and MLC1 when NE was added to the LPS-treated PHCC. In contrast, troponin I showed no decrease in protein levels in the presence of LPS. Several trials need to be done in order to confirm that troponin I protein levels are unaffected by

LPS treatment in PHCC. According to Cowan et. al., LPS is able to be transported inside the cell using sCD14 proteins allowing the cell to engulf LPS. The LPS vesicle then travels to the Golgi via retrograde transport where it aggregates (Cowan et al., 2001). LPS potentially prohibits Golgi processing of proteins which could lead to altered protein expression. Potentially, the proposed mechanism could be the reason contractile protein fluorescence was diminished in the presence of LPS. Once in the cell, LPS also associates itself with the contractile protein apparatus, which also suggests how LPS can effect early cardiac contraction (Cowan et al., 2001). NE provided a beneficial effect to the contractile proteins as the expression of the proteins increased when treated with NE. To better understand how NE specifically increases contractile protein expression, RT-PCR experiments need to be performed to measure if gene expression of the contractile proteins is increased in order to nullify the effect LPS has on the proteins.

In summary, our findings could add to the overall the understanding behind the mechanisms of cardiovascular disease. PHCC did not exhibit any changes in viability in the presence of LPS but the expression of contractile proteins was altered. The decreases in the contractile proteins were reduced when NE was added to the media suggesting that NE may play a critical role in the regulation of cardiomyocyte function.

6 CONCLUSIONS

The current series of experiments provides the following key results:

1. LPS (1-100ng) did not affect the viability of PHCC and the expression of TNF-alpha levels.
2. LPS at higher doses (100µg) produced a decrease in the levels of contractile proteins (F-actin, troponin I and tropomyosin).
3. NE ameliorated the LPS-induced reduction of fluorescence in the contractile proteins.

These data demonstrate LPS affect the expression of contractile proteins in PHCC without affecting the cell viability. The PHCC cell line is not sensitive to LPS-induced activation of cytokines and induction of apoptosis. At extremely high doses, however, LPS produced depressed levels of contractile proteins and this effect is reversed by NE. It appears that LPS induced decrease in protein levels of myofibrillar and contractile proteins in PHCC might not be due to induction of apoptosis.

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