Ministry of Higher Education
And Scientific Research
University of Kufa
College of Medicine
Department of Pharmacology and Therapeutic

Effect of N-acetyl cysteine and TAK 242 on Sepsis induced Myocardial Injury: Down-Regulation of MMP-2 Pathway in Mice

A Thesis
Submitted to the Council of the College of Medicine and the Committee of Postgraduate Studies, University of Kufa in Partial Fulfillment of the requirements for the Degree of Master of Science in Pharmacology and Therapeutics

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بسم الله الرحمن الرحيم

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

يَزْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ

الآية 11

سورة المجادلة

سورة المجادلة الآية 11
This thesis is dedicated to my parents **Dr. Azher** and **Dr. Ibtissam** who instilled in me the virtues of perseverance and relentlessly encouraged me to strive for excellence. Your affection, encouragement, and prays of day and night make me able to get such success and honor.

To my husband **Dr. Rasoul** who provided me everything to achieve success, supported me in every step and directed me towards that nothing is ever impossible. Your love, support and encouragement have motivated me in every step; I am forever grateful.

To my lovely and sweet daughter **Malak** who gave me the strength to complete my study.

*Safa Azher Al-khalidy*  
2016
First...

Praise be to our Almighty Allah, the Gracious Who gives me the power and motivation and inspiring me with strength to perform the present work.

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Safa Azher Al-khalidy
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<td>AGTRL-1</td>
<td>Angiotensin II receptor like -1</td>
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<td>ATP</td>
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<td>cAMP</td>
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<td>CD-14</td>
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<td>COP</td>
<td>Cardiac output</td>
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<td>cTn-I</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ECM</td>
<td>Extra-cellular matrix</td>
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<td>ECMO</td>
<td>Extracorporeal membrane oxygenation</td>
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<td>EF%</td>
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<td>ELISA</td>
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<td>iNOS</td>
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<td>LBP</td>
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<td>mRNA</td>
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<tr>
<td>TAK 242</td>
<td>Ethyl-(6R)-[N-(2-chloro-4-fluorophenyl] sulfamoyl] clohex-1-hene-1-carboxylate</td>
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<tr>
<td>TIMPs</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
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<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
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<tr>
<td>TLR-4</td>
<td>Toll like receptor -4</td>
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<td>Tn-C</td>
<td>Troponin C</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<td>Tn-T</td>
<td>Troponin T</td>
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Abstract

Background:

Myocardial injury is an early and serious complication of severe sepsis and septic shock, which occurs in 40% - 50% of septic patients. Myocardial injury is one of the major determinants of poor prognosis in septic patients who display ventricular dilation, reduced ejection fraction and contractility with decreased sensitivity to catecholamines and fluid resuscitation. In the intensive care unit, about 60% of severe septic patients exhibit cardiac injury and the mortality for those patients range from 70-90%. In contrast, the mortality in patients who are not showing any signs of myocardial injury due to sepsis is only 20%.

Matrix Metalloproteinase-2 (MMP-2 also known as gelatinase A) belongs to the MMPs family and contributes to the cardiovascular injury and remodeling in both physiological and pathological conditions due to its ability to degrade extra- and intra-cellular proteins. A variety of cells, including cardiac myocytes, synthesize MMP-2 as an inactive enzyme which is termed as a pro MMP-2 or zymogen and its activation occurs by proteolytic cleavage of the amino terminal domain or by conformational changes induced by denaturing or oxidative stress molecules such as peroxynitrite. In sepsis, increased production of pro-inflammatory cytokines depresses myocardial contractile function by increasing MMP-2 expression which results in degradation of troponin-I, myocin light chain-1, α-actinin and titin which are essential proteins for cardiomyocytes contractility thereby results in decreased myocardial contractility. NAC and TAK 242 are used in order to investigate their effects in attenuation of myocardial injury during sepsis.
**Objective:**

This study is undertaken to investigate the effect of pre-treatment with NAC and TAK 242 in attenuation of sepsis induced myocardial injury through down regulation of MMP-2 pathway.

**Materials and Methods:**

A total of 60 Adult male Albino-Webster mice were used in this study. Their weights ranged from 30 to 35 g. The study was conducted at Kufa University, College of Medicine, Department of Pharmacology and Therapeutics from November 2015 to April 2016. Mice were randomly divided into 5 groups (12 mice in each group). **Group 1 sham group**, which was subjected to anesthesia and laparotomy with no cecum ligation or puncture and served as the surgical control group, **group 2 CLP group**, which was subjected to cecal ligation puncture surgery and served as poly-microbial sepsis control group, **group 3 NAC pretreated group**, which was treated with 150 mg/Kg i.p. of NAC 1 hour before they were subjected to cecal ligation puncture surgery, **group 4 TAK 242 pretreated group**, which was treated with 3mg/Kg i.p. of TAK 242 1 hour before they were subjected to cecal ligation puncture surgery, **group 5 Vehicle pre-treated group**, which was given 10ml/kg i.p. 1 hour before they were subjected to cecal ligation puncture surgery.

At the end of experiment (24 hours after cecal ligation puncture surgery), the left ventricular function was analyzed by using a microcatheter system with the aid of PVAN software (Millar Instruments) then blood samples were collected from the heart then the hearts were excised and divided in to two parts: The apical side was fixed in 10% neutral buffered formalin for histological examination and the basal side was homogenized. Plasma level of cardiac
troponin I (cTn-I) and levels of proinflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6 and MCP-1) both in plasma and myocardium were analyzed by enzyme-linked immunosorbent assay (ELISA). Furthermore, the cardiac MMP-2 was measured by qRT-PCR and the pathological changes and cells injury in the myocardium were examined using Hematoxylin and Eosin staining.

**Results:**

Compared with the sham group, levels of plasma and myocardial TNF-α, IL-1β, IL-6, MCP-1 and plasma levels of cTn-I were significantly (p<0.05) increased in CLP and vehicle groups. Poly-microbial sepsis significantly (p<0.05) increased cardiac MMP-2 expression and led to impairment of left ventricular function expressed as a significant reduction in ejection fraction, cardiac output and left ventricular end systolic pressure values as well as a significant elevation in heart rate and left ventricular end diastolic pressure values. These changes are consistent with histopathologic examination which revealed a marked myocardial injury with the development of contraction bands and polymorphonuclear leukocytes infiltration besides interstitial edema and localized extravasation of red blood cells as compared to sham group. All these changes were counteracted by administration of NAC and TAK 242 which include reduction of plasma and myocardial TNF-α, IL-1β, IL-6, MCP-1 and plasma levels of cTn-I with decreased cardiac MMP-2 expression and improvement of left ventricular function expressed as a significant increase in ejection fraction, cardiac output and left ventricular end systolic pressure values as well as a significant reduction in heart rate and left ventricular end diastolic pressure values. These changes are consistent with histopathologic examination which revealed a mild to moderate myocardial injury as compared to CLP group.
Conclusions:

The results of the present study reveal that NAC and TAK 242 have been shown to decrease sepsis-induced myocardial injury through interfering with inflammatory mediators and down regulation of MMP-2 signaling pathway. Additionally, prophylactic use of these drugs improves the left ventricular function.
Chapter One

Introduction & Literature Review
1. Introduction

1.1. Sepsis Induced Myocardial Injury

Sepsis induced myocardial injury is the most common finding leading to increase the morbidity and the mortality in sepsis patients. It is defined as a global (systolic and diastolic) injury of both left and right sides of the heart (Antonucci et al., 2014). It is also recognized as the inability of the heart to meet the increment in the metabolic demands during sepsis (MacLean et al., 1967) which occurs due to the functional and structural injury in the myocardium with or without lowered cardiac output (Mueller-Werdan et al., 2006) and it is characterizing by dilatation of the left ventricle and reduction in ejection fraction (Sato and Nasu, 2015). It occurs when the sepsis patients fail to increase their cardiac output in response to resuscitation (Weisel et al., 1977). Therefore; the occurrence of multiple organs injury syndrome in the sepsis patients is greatly due to myocardial injury which leads to a decrease in the cardiac output, so these results in vital organs hypoperfusion, reduction in oxygen and nutrition supply of the tissues and immunity suppression; finally organs injury occurs (Kumar, Haery and Parrillo, 2000).

Myocardial injury is a characteristic manifestation of sepsis and septic shock, which occurs in 40 % - 50 % of septic patients (Fernandes & Assuncao, 2012). In the intensive care unit, about 60% of severe sepsis patients exhibit cardiac injury and the mortality for those patients ranges from 70-90%. In contrast, the mortality in patients who are not showing any signs of myocardial injury due to sepsis is 20% only (Celes et al., 2012). There is great increment in the mortality of septic patients with left and right ventricles injury (Kumar et al., 2000).
1.2 Characteristic Features of Myocardial Injury during Sepsis

Myocardial injury is one of the major determinants of poor prognosis in sepsis patients with ventricular dilation, reduction in ejection fraction and contractility with decreased sensitivity to catecholamines and fluid resuscitation (Vieillard-Baron et al., 2008; Francisco et al., 2011). Human and experimental studies (Ren et al., 2002; Merx et al., 2005) have clearly confirmed that reduced contractility and impaired myocardial performance are important factors that cause myocardial injury in sepsis which can present as left ventricular diastolic and systolic injury, right ventricular injury or biventricular injury (Pulido et al., 2012). The heart function in sepsis was first demonstrated in 1984 by using radionuclide cineangiography (Parker et al., 1984). In a series of 20 patients, a dilatation of the left ventricle with a reduction in ejection fraction, i.e., increased left ventricular end-diastolic volume is demonstrated. Strangely, survivors show a more pronounced left ventricular dilatation and ejection fraction reduction, and reversibility within 7-10 days. In contrast, non-survivors, held their initial ejection fraction, appear unable to dilate their left ventricle. The left ventricular systolic injury incidence is variable which ranges from 18-29 % in first 6 hours, then at 12 hours increases to 46% and reaching 65% at the end of the first day (Vieillard-Baron, 2011). Myocardial injury during sepsis has three characteristic features:

1. Left ventricular dilatation with normal or low filling pressure. This possibly happens due to abnormal increase in the left ventricular end-diastolic volume leading to increase in the LV compliance (Sato and Nasu, 2015).
2. Reduction in ejection fraction (Hunter and Doddi, 2010).
3. Reversible which resolves within 7–10 days in survivors (Kumar et al., 2000).
1.3 Pathogenesis of Myocardial Injury in sepsis

The mechanism of sepsis induced myocardial injury remains dispute. Potential mechanisms include the change in circulating blood volume; a direct inhibition of myocyte contractility by cytokines (TNF-α, IL-1β); abnormal nitric oxide and reactive oxygen species (ROS) signaling; mitochondrial injury; abnormal excitation-contraction coupling; and reduced calcium sensitivity at the myofibrillar level and blunted β-adrenergic signaling. So, the pathogenesis of sepsis-induced myocardial injury is now regarded as the result of a complex interaction between systemic factors and molecular, metabolic and structural alterations.

1.3.1. Circulatory Changes in Sepsis Induced Myocardial Injury

Intravascular volume reduction and vasodilation that occur early during sepsis and septic shock are characteristic features of circulatory abnormalities. Consequently the heart under-filling leads to decrease in cardiac output which leads to an imbalance in oxygen consumption in various organs that is usually reversed by fluid resuscitation (Rivers et al., 2001). So adequate fluid resuscitation in experimentally septic animals results in hyper-dynamic state or warm sepsis which associates with high cardiac output and low systemic vascular resistance, while septic hypo-dynamic state or cold sepsis occurs in inadequately resuscitated animals and associates with low cardiac output (Rabuel and Mebazaa, 2006). However, in animal models, there is no cellular hypoxia (Hotchkiss et al., 1991). So this makes this mechanism appear to be of low importance in the onset of myocardial injury during sepsis.
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1.3.2 Microvascular Changes in Sepsis Induced Myocardial Injury

During sepsis, the microcirculation undergoes several changes, with blood flow misdistribution and endothelial disruption (Hinshaw, 1996). These findings may be caused by non-occlusive intravascular deposits of fibrin in the microvasculature and endothelial swelling (Solomon et al., 1994). Also there is migration and activation of circulating neutrophils into the interstitium (Madorin et al., 2004). Where these cells may enhance the intra-cardiac inflammation induced by sepsis, and contribute to an enhanced vascular leakage, which also describes the weakness in the heart function and compliance secondary to myocardial edema (Chagnon et al., 2006).

1.3.3 Autonomic Dysregulation in Sepsis Induced Myocardial Injury

Autonomic dysregulation occurs in experimentally septic animals (Cariou et al. 2008) and includes unresponsiveness to catecholamines, although there are elevated levels of these substances in the circulation. The dysregulation is due to decreased density of myocardial adrenergic receptors, signal transduction disruption and inhibitory G-protein over expression. Additionally apoptosis of glial cells and neurons in the autonomic centers, which are responsible for the cardiovascular system control, contribute to the autonomic dysregulation during sepsis and may be induced by chemical mediators that cause an insufficient autonomic control of the circulatory system in septic patients (Sharshar et al., 2004).
1.3.4 Metabolic Changes in Sepsis Induced Myocardial Injury

During sepsis, there are marked metabolic alterations which include intra-cardiomyocyte accumulation of glycogen and lipids in non-survivors septic patients (Rossi et al., 2007) and in experimental animals (Levy et al., 2005). The sepsis patients’ hearts always have a net lactate extraction, while glucose, free fatty acids and ketone bodies uptake are decreased (Dhainaut et al. 1987).

During severe sepsis, oxygen utilization by cells is less, so they are suffering from a defect in oxygen delivery to the tissues. Because 90% of total oxygen consumption in the body is used by mitochondria for adenosine triphosphate production, mitochondria may play an essential role in the pathogenesis of organ injury induced by sepsis (Rudiger et al., 2007).

1.3.5 Mitochondrial Injury in Sepsis Induced Myocardial Injury

Several studies identify that myocardial mitochondrial injury occurs in both septic patients and experimental animal models and correlates to the degree of severity and prognosis (Drosatos et al. 2013; Vanasco et al. 2014). The increased production of superoxide and nitric oxide in mitochondria in addition to the depletion of intra-mitochondrial antioxidants during sepsis may negatively inhibit the oxidative phosphorylation and ATP generation (Brealey et al. 2002). Interestingly, mitochondrial DNA appears to be more sensitive to the damage induced by LPS than nuclear DNA (Suliman et al., 2004).
1.3.6 Apoptosis and Cell Death in Sepsis Induced Myocardial Injury

There is highly increasing evidence about the role of apoptosis in sepsis induced myocardial injury (Buerke et al., 2008). During sepsis, there is stimulation of many caspase enzymes, the apoptosis effectors, and release of mitochondrial cytochrome C (Kumar et al., 2005). Some strategies inhibit apoptosis in experimental animal models of sepsis improving contractile function (Neviere, 2001). As a summary, apoptotic cell death of the myocardium is a seldom event during sepsis and inadequate to account for the observed functional depression. This condition is reversible which also supports functional rather than anatomical abnormalities (Rudiger et al., 2007).

1.3.7 Contractile Apparatus Injury in Sepsis Induced Myocardial Injury

1- Calcium transport

In cardiomyocytes isolated from animals, it is found that both endotoxin (Abi-Gerges et al., 1999) and cytokines (Liu and Schreur, 1995) change or depress the L-type calcium channel, possibly through alteration in autonomic regulation of this channel. This results in a reduction of intracellular calcium concentration and a decrease in the fiber contractility. In addition, ATP-dependent potassium channels are opened by endotoxin, leading to shortening of the action potentials and decreasing calcium overload (Buckley et al., 2006). In a pig sepsis model, there is a reduction in calcium current with subsequent shortening of cardiac repolarization (Stengl et al., 2010). During experimental sepsis models, the density of the ryanodine receptor on the sarcoplasmic reticulum, which is responsible for triggering calcium release, is decreasing and resulting in attenuation of calcium release from the sarcoplasmic reticulum (Dong et al., 2001). Attenuation of
calcium uptake and release from sarcoplasmic reticulum stores with decreasing in the sensitivity of calcium channel are all associating with sepsis induced myocardial injury (Wu et al., 2001).

2- Myofibrillar Injury

During sepsis, reduction in the myofilaments sensitivity to calcium appears to be associated with increment in the cardiomyocytes length and ventricular distensibility (Tavernier et al., 2001). Moreover, there is a disruption area in the actin-myosin contractility apparatus in the hearts of septic patients (Rossi et al., 2007) which can be due to the enhanced matrix metalloproteinase activity, because this enzyme can destroy both the contractility apparatus and cytoskeleton (Wang et al., 2002, Gao et al., 2003). So reversal of these structural changes may occur slowly, especially if there is de novo protein synthesis. In animal models of sepsis, there is alteration in the calcium sensitivity of myofibrillar proteins (Wu et al., 2001).

3- β-Adrenergic Signaling Pathway

Activation of β-adrenergic receptors on the myocardium results in increasing of heart rate and contractility. So when these receptors are over stimulated or occupied for long time, this leads to the damage of the myocardium by calcium overload and consequent cell necrosis (Opie, 2004). Furthermore, there is a marked disruption of the signal transduction in the myocardium following β-adrenoceptor stimulation. In experimental sepsis model, there is reduction in the stimulatory G-proteins levels and enhanced expression of inhibitory G-protein (Matsuda et al., 2000; Wu et al., 2003). These events are presumably to inhibit the adenylyl cyclase activity, resulting in reduced cyclic adenosine monophosphate
(cAMP) levels inside the cell, paralyzing the cardiomyocyte. So, it remains elusive whether a blunted stimulation of β-adrenergic receptor, disruption of the downstream signaling cascades, or a combination of both which are associated with myocardial injury in sepsis.

1.3.8 Inflammatory Signalling Pathway in Sepsis Induced Myocardial Injury

1- Nitric Oxide and Peroxynitrite

Nitric Oxide synthesis occurs by cleaving of L-arginine to L-citrulline by three nitric oxide synthase isoforms within the myocardium: inducible, neuronal and endothelial nitric oxide synthase (Schulz et al., 2005). Neuronal and endothelial nitric oxide synthase are normally expressed in the myocytes and produce nitric oxide during myocytes contraction due to the regulation of calcium-calmodulin. During sepsis, there is overproduction of nitric oxide due to endothelial activation by pro inflammatory cytokines, resulting in overstimulation of inducible nitric oxide synthase (iNOS) (Greer, 2015). Therefore, non-specific inhibition of nitric oxide synthase reverses the changes in cardiac output and stroke volume that occurs after injection of LPS (Hwang and Yeh, 2002). In sepsis patients, infusion of methylene blue which is a nonspecific inhibitor of nitric oxide synthase improves stroke volume, left ventricular stroke work and mean arterial pressure, and reduces the need for inotropic agents, but it does not change the outcome (Kirov et al., 2001).Moreover, some evidence reveals that most of the nitric oxide cytotoxicity is really attributed to peroxynitrite produced from the interaction between nitric oxide and superoxide anion, which is another free radical. Peroxynitrite reacts directly, via oxidative reactions or indirectly with lipids,
proteins and DNA, and can be greatly cytotoxic. During experimental sepsis, Peroxynitrite, rather than nitric oxide itself, weakens the contractility of the muscle through its ability to denature proteins, disturb calcium flux, and suppress mitochondrial respiration (Pacher et al., 2007).

2- Toll-like Receptors-4

Toll like receptors (TLR) serve as pattern recognition receptors that generate innate immune response to pathogens by activating a cascade of pro-inflammatory events (Kimmoun and Levy, 2011). TLR-4 is a member of TLR family and classifying as transmembrane type-1 receptors with leucin-rich repeat extracellular motifs and a preserved cytoplasmic Toll/IL-1 receptor (TIR) domain (Kawai and Akira, 2010). Cardiomyocytes express TLR-4 like dendritic cells, which enable them to respond to endanger signals and mediate local inflammatory responses (Baumgarten et al., 2001). During sepsis, TLR-4 must find on macrophages, and on neutrophils to a lesser extent, in order to induce myocardial injury (Thomas et al., 2003). When mice undergo LPS challenge, the rapid and over activation of NF-κB, with consequent increase of TNF-α and IL-1β mRNA expression in cardiomyocytes are greatly ameliorating in TLR4-mutant mice (Baumgarten et al., 2001). Recognition of LPS begins with the binding to lipopolysaccharide-binding protein (LBP), an acute phase protein. LBP then facilitates the transfer of LPS to CD14. After binding of LPS to the TLR-4-MD-2 complex, dimerization of this complex happens, this result in the recruitment of adapter molecules and initiation of signaling pathway (Fitzgerald et al., 2004, DeMarco et al., 2011). Subsequent to LPS binding to TLR-4-MD-2, two pathways are activated (early vs. delayed). The early pathway leads to activation of nuclear factor κB (NF-κB) which induces production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6. The other pathway results in delayed activation of NF-κB and leads to
production of IFN-β and IFN-inducible genes (Akira and Takeda, 2004). After TLR-4 activation, the NF-κB pathway has a key role in decreased contractility and inflammatory response of cardiomyocytes (Boyd et al., 2006).

3- Role of Proinflammatory Cytokines

In sepsis, the concept of circulating myocardial depressant factors is first proposing in more than 50 years (Lefer, 1970). The existence of a cardio-depressant substance is confirming by the incubation of isolated rat cardiomyocytes with serum from septic shock patients, leading to decrease the velocity and amplitude of cardiomyocytes shortening (Parrillo et al., 1985). The activation of TLR-4 leads to nuclear translocation of NF-κB which induce the production of cytokines and adhesion molecules in immune cells and non-immune cells such as endothelial cells, fibroblasts, and cardiomyocytes. These cytokines play critical roles in myocarditis and cardiomyopathy (Feng and Chao, 2011). TNF-α, IL-1β and IL-6 are the main inflammatory mediators that contribute to myocardial injury in sepsis, in which activated macrophages secrete TNF-α, but recent studies show that cardiac myocytes secrete TNF-α and represent the major source of TNF-α in the myocardium (Grandel et al. 2000, Peng et al. 2003). It is noteworthy that cardiomyocytes are able to produce TNF-α, IL-1β, IL-6 during endotoxemia, sepsis, and burn injury (Maass et al., 2002; Garner et al., 2003; Carlson et al., 2005)

TNF-α is a pro-inflammatory cytokine and its expression in high levels in the heart during sepsis leads to myocardial injury (Zhang, 2011). TNF-α causes calcium dysregulation, oxidative stress, direct cytotoxicity, up regulation of other myocardial suppressing cytokines (e.g., IL-1β), disruption of excitation-contraction
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coupling, and the induction of cardiomyocytes apoptosis (Zhao et al. 2006). In animal model, the administration of recombinant TNF-α leads to the existence of fever, hemodynamic changes, lactic acidosis, and even death. Several studies in humans and other animals using anti-TNF-α antibodies show a rapid improvement in cardiac contractility, with no reduction in mortality (Bujak and Frangogiannis, 2009).

Monocytes, macrophages, and neutrophils secrete IL-1 in response to TNF-α, which is a fever inducing substance and represents the center of the inflammatory response. It has two sub unites that are IL-1α and IL-1β (Miller et al., 2006). IL-1β plays an important role in myocardial injury during sepsis, both alone and synergistically with TNF-α. It is possibly that there is a relation between TNF-α/IL-1 and iNOS, creating a negative inotropic effect on the heart (Bujak and Frangogiannis, 2009).

IL-6 is a pro-inflammatory cytokine that is expressed by different cell types including macrophages and T cells that stimulate immune response by secreting IL-6 during infection and other causes of inflammation. IL-6 also is secreted by endothelial cells, mast cells and other type of cells. In addition, it is a sensitive marker of tissue damage, and its level is directly related to endotoxin production during infectious diseases (Pallua and Heimburg 2003). The presence of IL-6 induces signaling events in vitro by using isolated ventricular cardiac myocytes from rat as a model of myocardial contractility in whole blood taken from meningococcal septic children (Pathan et al., 2011).
1.4 Role of Matrix Metalloproteinase-2 (MMP-2) in Myocardial Injury during sepsis

The collagen network is a crucial component of the heart’s extracellular matrix (ECM) where it works to secure and support cardiomyocytes in a proper alignment required for coordinated contraction and it helps in determining heart compliance. The ECM is mainly composed of collagen with smaller proportion contributed by elastin, laminin and fibronectin. The highly organized network provided structural integrity between adjacent cardiomyocytes and coordination to overall myocyte shortening. In normal myocardium, only about 2-4% of the myocardium is reported to be comprised of collagen. Yet, even small changes in collagen concentration have been shown to mediate drastic effects on the heart’s mechanical properties (Brower et al., 2006). Modulation of cardiac extracellular matrix occurs by many members of the large matrix metalloproteinase (MMP) family. So MMPs has an important mechanistic focus in the evolution of left ventricular failure in both ischemic and non-ischemic disease (Sierevogel et al., 2003; Lindsey, 2004; Spinale et al., 2002). The MMPs are a family of zinc-dependent, calcium-containing endopeptidases. They have a potential role in the remodeling of the extra-cellular matrix during different physiological processes, including angiogenesis (Roy et al., 2006) and embryogenesis (Vu and Werb, 2000) in addition to their role in pathological processes such as tumor metastasis (Deryugina and Quigley, 2006), inflammation and arthritis (Mohammed et al., 2003), endotoxemia (Rhee et al., 2007) and cardiovascular diseases (Castro et al., 2008; Chung et al., 2008).

Matrix metalloproteinase (MMP-2 also known as gelatinase A) belongs to the matrix metalloproteinase (MMPs) family and contributes to the cardiovascular injury and remodeling in many diseases due to its ability to degrade extra- and
in intracellular proteins (Chow et al., 2007; Raffetto and Khalil, 2008). It has the regard of substantial interest, as excessive MMP-2 activation results in the progression of heart failure (Iwanaga et al., 2002; Stewart et al., 2003; Sakata et al., 2004). A variety of cells, including cardiac myocytes, synthesize MMP-2 as an inactive enzyme which termed as a pro MMP-2 or zymogen and its activation occurs by proteolytic removal of the amino terminal domain (Galis and Khatri, 2002) or, alternatively, by S-glutathiolation of a cysteine in this propeptide, by reaction with peroxynitrite resulting in 72 kDa active MMP-2 (Viappiani et al., 2009). Activation of MMP-2 is very important for its proteolytic activity both inside and outside the cell. Regulation of MMP-2 activity occurs at different levels including transcriptional, post transcriptional, post translational modifications, and by interaction with its endogenous tissue inhibitors (TIMPs) (Schulz, 2007). In cardiac cells, expression of MMP-2 can be actively up-regulated in response to hypoxia, angiotensin II, endothelin-1 or IL-1β (Bergman et al., 2003; Alfonso-Jaume et al., 2006). Additionally, in cardiac micro-vascular endothelial cells, MMP-2 protein and mRNA levels can be stimulated by pro-inflammatory cytokine (Mountain et al., 2007). Expression of MMP-2 occurs in cardiomyocytes, endothelium, fibroblasts and vascular smooth muscle cells (Wang et al., 2002).

In normal cardiomyocytes, MMP-2 locates in discrete subcellular compartments, including the mitochondria (Wang et al., 2002), thin and thick myofilaments of the cardiac sarcomere (Sawicki et al., 2005), nuclei (Kwan et al., 2004), cytoskeleton (Sung et al., 2007) and caveolae (Chow et al., 2007). In these cells, oxidative stress results in activation of MMP-2 which colocalizes and causes cleavage of target proteins including troponin I (Wang et al., 2002), myosin light chain-1 (Sawicki et al., 2005), α-actinin (Sung et al., 2007) and titin (Cho et al., 2010) proteins which are essential for cardiac muscle contraction and cause acute,
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reversible contractile injury (Schulz, 2007). Calponin-1, which is a regulator of vascular smooth muscle tone with similarities to troponin, is a new target of MMP-2 which cleaves it and contributes to endotoxemia-induced vascular hypocontractility (Castro et al., 2012).

MMP-2 has a role in multiple acute biological processes independent on its effects on extracellular matrix proteins which includes platelet activation (Sawicki et al., 2005), vascular tone regulation (Fernandez-Patron et al., 1999) and myocardial stunning injury immediately after ischemia/reperfusion (Cheung et al., 2000). Actually, several reports reveal that MMP-2 does not exclusively degrade extracellular matrix components (McCawley and Matrisian, 2008). During ischemia/reperfusion, MMP-2 is responsible for disruption of the coronary endothelium with subsequent increasing in its permeability. The increment of MMP-2 activity results in cardiac injury (Fert-Bober et al., 2008). Pro-inflammatory cytokines results in activation of MMP-2 and a decrease in tissue inhibitor of matrix metalloproteinase-4 (TIMP-4) in the heart. So during acute heart failure, pro-inflammatory cytokines induce MMP-2 and result in proteolysis of troponin I. MMPs inhibition may be a novel pharmacological strategy for the treatment of acute inflammatory heart disease (Gaoa et al., 2003). So, inhibition of MMP-2 activity with non-selective agents is associated with improvement of left ventricle injury, modulation of chronic heart failure models (Sakata et al., 2004; Chen et al., 2004) and improvement of hemodynamic function after ischemia/reperfusion injury with prevention of left ventricle remodeling several weeks later (Villareal et al., 2003).
1.5 Troponin I (cTn-I) as a Marker for Cardiac Injury during Sepsis

Troponins are regulatory contractile proteins of the thin actin filaments of the cardiac muscle (Ammann et al., 2001) which are also responsible for regulating the contractility of skeletal muscle while smooth muscle cells do not have troponins (Kim et al., 2005). The troponin complex is attached to tropomyosin and has three subunits: troponin C (Tn-C), troponin T (Tn-T), and troponin I (Tn-I). Tn-C contains the binding sites for calcium that helps initiate contraction and detects calcium concentration. Tn-T is a structural protein that attaches the troponin complex to tropomyosin. Tn-I is the inhibitory subunit that inhibits the interaction of myosin with actin (Galinska-Rakoczy et al., 2008).

cTn-I and cTn-T are releasing due to cell injury in the myocardium, and are highly sensitive and specific markers of myocardial damage. cTn-I is a very sensitive plasma marker of metabolic or physical myocardial injury, myocardial ischemia, or necrosis with a cardiac specificity of 100% (Willott et al., 2010). A significant correlation between the elevated plasma level of cTn-I and the decrease in ejection fraction is confirming in a study of 37 patients with septic shock. In which 16 patients (43%) are with an elevated serum level of cTn-I and a significantly lower EF (Chen et al., 2004).
1.6 Therapeutic Strategies for Myocardial Injury during sepsis

No specific therapy for sepsis induced myocardial injury. Recently, general therapeutic strategies include infection control, hemodynamic support, host response modulation and critical care support (De Kock et al., 2010). Septic shock patients need the use of inotropes and/or vasopressors to maintain adequate mean arterial pressure, oxygen delivery, and cardiac contractility (Takasu et al., 2013). They also need an adequate fluid infusion support to maintain perfusion pressures and adequate blood flow for regional and global demands.

1.6.1 Non pharmacological approach

1- Intra-aortic balloon Pump or counterpulsation (IABP)

IABP is a mechanical device that increases myocardial oxygen perfusion while at the same time increasing cardiac output. It reduces the vasopressor dose and prolongs survival time in a canine model of severe septic shock (Solomon et al., 2009). It consists of a cylindrical polyethylene balloon that sits in the aorta, approximately 2 centimeters from the left subclavian artery and counter pulsates (Overwalder, 1999). That is, it actively deflates in systole, increasing forward blood flow by reducing afterload through a vacuum effect. It actively inflates in diastole, increasing blood flow to the coronary arteries via retrograde flow as shown in figure (1). These actions combine to decrease myocardial oxygen demand and increase myocardial oxygen supply (Landoni et al., 2012). A computer-controlled mechanism inflates the balloon with helium from a cylinder during diastole, usually linked to either an electrocardiogram (ECG) or a pressure transducer at the distal tip of the catheter. It may be used as a maintenance therapy.
for septic patients with myocardial injury. IABP needs 3-24 hours to be fully operated; therefore the insertion should be early (Christoph et al., 2008). Rare complications are associated with the use of IABP and may increase in septic shock patients with renal injury or disseminated intravascular coagulation. So the perfect patient to use the IABP must have a severe myocardial depression and a not highly reduction in systemic vascular resistance (Wendan, 2009).

Figure (1): Insertion and Action of IABP
2- Apelin therapy

Apelin is G-protein coupled endogenous ligand related to angiotensin-1 receptor, also called angiotensin II receptor like-1 AGTRL1. It is highly potent positive inotropic peptide, supposing that a decrease in the endogenous concentration of Apelin plays an essential role in the heart failure development (Berry et al., 2004). In an experimental rat’s model of sepsis, there is a decrease in the plasma and myocardial Apelin and AGTRL1 concentrations and that exogenous apelin administration results in improvement of cardiac injury, with more hemodynamic stability and less cytokines release such as MCP-1 and IL-8 (Pan et al., 2010).

3- MSCs (Mesenchymal stem cells)

The MSCs infusion is a novel treatment for myocardial injury during sepsis. In a murine experimental model, MSCs modulate the systemic inflammatory response by decreasing the TNF-α, IL-1β and IL-6 levels which are produced by host macrophages and lessen the myocardial injury during sepsis. Also there is an increase in the serum levels of anti-inflammatory cytokines (IL-10) (Weil et al., 2010).

4- ECMO (Extracorporeal membrane oxygenation)

ECMO is an extracorporeal technique of providing both cardiac and respiratory support to persons whose heart and lungs are unable to provide an adequate amount of gas exchange to sustain life. This intervention has mostly been used on children, but it is seeing more use in adults with cardiac and respiratory failure. ECMO works by removing blood from the person’s body and artificially removing the carbon dioxide and oxygenating red blood cells as shown in figure (2). Generally it is only used in the later treatment of a person with heart or lung failure.
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as it is solely a life sustaining intervention (Van Meurs et al., 2005). There are several forms of ECMO, the two most common of which are the veno-arterial (VA) and veno-venous (VV). In both modalities, blood drained from the venous system is oxygenated outside of the body. In VA ECMO, this blood is returned to the arterial system and in VV ECMO the blood is returned to the venous system (Madershahian et al., 2006). It is the last lifesaving therapy to unresponsive patients with sepsis induced myocardial injury. So this strategy may give time for antibiotics to work effectively and improve mortality. However, the experience of using this strategy in patients with septic shock is very limited. Additionally, the management of patients who need ECMO is very difficult. Thus, a good experienced team should use it in a specialized center (Combes et al., 2014).

Figure (2): ECMO Machine
1.6.2 Pharmacological approach

1- Vasoactive therapy

Adrenergic agonists are the first choice for treatment of hypotensive septic patients, they increase blood pressure by stimulating α-adrenergic receptors located in resistive arterioles (De Backer and Scolletta 2013). Several evidence suppose that norepinephrine is effective in the initial stages of sepsis induced myocardial injury but with long term may become deleterious due to sympathetic over activation (Francisco et al., 2011). Vasopressin and terlipressin are most widely used as salvage vasopressors in catecholamine resistant septic shock treatment (Wilson et al., 2005). In severe sepsis and septic shock, there is vasopressin deficiency with down regulation of its receptors that justify their use (Schmidt et al., 2008). Vasopressin is a highly potent vasopressor which acts directly on vasopressin-1 receptors and enhancing the effects of other catecholamines so enabling norepinephrine doses to be reduced (Serpa et al., 2012). Terlipressin acts more specifically on vasopressin-1 receptors and more potently increases the blood pressure without rebound hypotension. The use of each drug is usually according to local availability (Lange et al., 2008).

2- Metabolic modification

Sepsis causes profound metabolic alterations and exposes vital organs to high risk of energy failure (Rudiger and Singer, 2013). Several strategies explain the ways in order to reduce the cellular energy crisis. For instance, pyruvate substitution is a provider of cellular ATP. In the Krebs cycle, pyruvate metabolism occurs under aerobic conditions while in anaerobic conditions pyruvate metabolizes to lactate at a lower ATP yield. Therapeutic pyruvate administration increases the energy availability in the heart and results in improvement of
intracellular calcium handling (Hasenfuss et al., 2002). In addition pyruvate has specific immunomodulatory properties that may have an important role. Mitochondrial injury occurs during sepsis which affects complex I of the respiratory chain (Brealey et al., 2004; Singer, 2007). Succinate is a complex II substrate and can efficiently bypass an injured complex I, so it improves mitochondrial oxygen utilization and ATP production (Protti et al., 2007). Furthermore, insulin transports glucose into the cardiac myocytes and enhances cardiac inotropy (Engebretsen et al., 2011). First experiments with high insulin doses in endotoxemic pigs are promising (Holger et al., 2010). However, this concept needs further research before can be used to septic patients.

3- Immuno-modulatory therapy

None of the available Immuno-modulatory strategies, proven to be highly effective in sepsis, design specifically to target myocardial injury. So strategies that preferentially target cardiac injury in sepsis may be a promising. For example, lipoteichoic acid, the main virulence factor in Gram-positive sepsis, causes cardiac injury via activation of myocardial TNF-α synthesis and stimulates coronary vascular disturbances by activation of thromboxane A-2 synthesis. So it contributes to cardiac injury and may be a cardiac-specific target (Grandel et al., 2005).

Several experimental animal studies in rats have shown that the dihydropyridines such as manidipine, nicardipine and especially amlodipine, apart from its effect on calcium channels, could reduce levels of proinflammatory cytokines and the expression of inflammation-relevant genes TNF-α and iNOS. Moreover, pretreatment with amlodipine weakens the myocardial inflammation induced by LPS (by decreasing the proinflammatory cytokines levels) and indirectly reduces the infiltration of inflammatory cells in the myocardium,
suggested a cardiac protective effect. So study the effects of dihydropyridines in an experimental sepsis model is interesting (Xiao-Qiang et al., 2009).

4- Cardioprotective therapy

Cardioprotective therapy is necessary to alleviate cardiac remodeling and injury (Ruegg, 2013). As β-adrenergic stress is a major factor in sepsis-induced myocardial injury, the use of β-blocking agents could be beneficial. However, this is still controversial as it may have potential negative inotropic effect in patients with sepsis induced myocardial injury. In sepsis patients, β-blockers as a classic drug to treat cardiac diseases are not only having the effects of preventing ischemia, decreasing oxygen demand, and TNF production (J Romero-Bermejo et al., 2011); they also reduce local and systemic inflammation (Marik and Flemmer, 2012). Moreover, ACEIs have positive effects on chronic inflammation in atherosclerosis. Though they cannot reverse cardiac remodeling, they can reduce acute cardiovascular events during inflammation (Tsikouris and Cox, 2003). Also angiotensin receptor blockers have the similar clinical effects as ACEIs though they work through different mechanisms, like increasing NO bioavailability and improving insulin sensitivity (Levine et al., 2012). Regarding inotropes only 10 to 20% of septic patients with myocardial injury need to receive inotropic agents to maintain adequate tissue perfusion. The majority of patients benefit from fluid infusion. However, dobutamine is the first choice when the indication of inotropic agents is necessary to optimize flow, cardiac output and improve hemodynamics (Fernandes et al., 2012). Dobutamine may modify the inflammatory immune response, probably by increasing the levels of TNF-α and IL-1 (Hartemink and Groeneveld et al., 2012).
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Milrinone is a phosphodiesterase III inhibitor has been used to stimulate the heart during sepsis (*Schmittinger et al.*, 2008). This approach is useful if the patient is treated with β-blockers, since the adrenergic agonistic effect of milrinone does not come from β-receptor activation but via a decreased cAMP degradation. Milrinone also decreases vascular tone, subsequently results in increasing the risks of arrhythmia and hypotension (*Cuffe et al.*, 2002).

Regarding that sepsis induced myocardial injury is also causing by a reduced myofilament response to calcium, so sensitization with levosimendan becomes an effective therapeutic choice. Levosimendan is a myofilament calcium sensitizer used in the management and treatment of acute and chronic heart failure. Unlike other inotropes, it improves cardiac function without stimulating the sympathetic nervous system and altering oxygen consumption. Levosimendan binds to troponin C at the N-terminal domain with high affinity at high calcium concentration which occurs during systole and inhibits troponin I resulting in prolonging the interaction between myosin and actin filaments (*Bangash et al.*, 2012). This sensitization decreases at low calcium concentration which occurs during diastole so diastolic relaxation remains highly unaffected. In contrast to other inotropes, levosimendan does not increase the oxygen consumption or cause arrhythmias. It also causes hyperpolarization of smooth muscle membrane by opening the ATP-sensitive potassium channels which leads to vasodilation. The drug is successfully used in both preclinical septic models (*Behrends and Peters*, 2003) and in septic shock patients (*Morelli et al.*, 2005).

Promising new inotropes that have been developed for heart failure patients are of highly interest for patients with sepsis induced myocardial injury (*Hasenfuss and Teerlink*, 2011) including Istaroxime which is a Na+/K+-ATPase inhibitor that activates sarcoplasmic reticulum calcium ATPase (SERCA) and exhibits
inotropic and lusitropic effects (Shah et al., 2009). Although initial clinical trials demonstrate a good safety profile but future studies are required before the drug can be regarded for clinical use.

Furthermore, omecamtiv mecarbil is a direct cardiac myosin-ATPase activator that increases the transition of myosin into the actin-bound force generating state (Malik et al., 2011). Importantly, Omecamtiv mecarbil improves cardiac contractility without increasing intracellular calcium transients or myocardial oxygen consumption (Hasenfuss and Teerlink, 2011). First clinical studies demonstrate that improvement of cardiac contractility is a dose-dependent without clinically relevant changes in diastolic function (Cleland et al., 2011). However, as omecamtiv mecarbil increases cardiac contractility by prolongation of systolic ejection time, cardiac filling may be attenuated at higher heart rates (as usually seen in septic patients).
1.7 Drugs Used In This Study

1- N-acetylcysteine (NAC)

![NAC Structure](image)

**Figure (3): NAC Structure**

N-acetylcysteine (NAC) also known as N acetyl-L-cysteine, NAC is a precursor of the amino acid L-cysteine and is a cell-permeable anti-oxidant in which the thiol (sulfhydryl) group gives antioxidant effects and can diminish free radicals (Frank Giorlando, 2011). Initially, NAC patented in 1960 and licensed for use in 1968. It is on the World Health Organization’s List of Essential Medicines, the medications needed in a basic health system. It is available as a generic medication and is not very expensive (Hitchings et al., 2014).

**Pharmacodynamics:**

NAC serves as a pro-drug to L-cysteine. L-cysteine is a precursor to the biologic antioxidant glutathione. Hence administration of NAC replenishes glutathione stores (Dodd et al., 2008). Mechanistically, NAC has antioxidant activity also it regulates the glutamatergic system. In concern to antioxidant activity, the cysteine component of NAC that consolidates with glutamate and glycine, all of them are glutathione precursors. Glutathione is a major endogenous antioxidant. In addition, it is the most generic cellular antioxidant in the body and it is essential for the immune system to exert its full potential (Berk et al., 2008).
Furthermore, NAC possesses some anti-inflammatory properties possibly due to inhibition of NF-κB and modulation of cytokine synthesis (Berk et al., 2008). In addition, it replenishes intracellular stores of glutathione, scavenges toxic ROS (via direct action and indirect action by increasing glutathione concentrations) and suppresses TNF-α production (Peristeris et al., 1992). At molecular level, NAC inhibits c-Jun N-terminal kinase, redox activating protein-1 and p38 MAP kinase activation (Zafarullah et al., 2003). NAC also suppresses neutrophils and macrophages activation, attenuates the adhesion of leukocyte–endothelial cell and capillary leakage, and modulates gene expression of TNF-α and IL-8 at the transcriptional level so blocks their release (Spapen, 2004).

N-acetylcysteine

\[
\begin{align*}
\text{ATP} & \rightarrow \text{Cysteine} & \text{Glutamate} \\
\text{ATP} & \rightarrow \gamma\text{-Glutamylcysteine} & \text{Glycine} \\
\text{Glutathione} & \rightarrow \text{Glutathione-S-transferase}
\end{align*}
\]

\[\downarrow \text{ROS}\]

Figure (4): NAC Mechanism of Action

Pharmacokinetics:

NAC is able to cross the placenta (Horowitz et al., 1997) and the blood brain barrier (Farr et al., 2003). The acetyl group attached to nitrogen in NAC encourages its absorption and permitting it to be much more readily absorbed than cysteine after ingestion (Sato et al., 1999). The peak plasma level of NAC is reached within 1 to 2 hours. The half-life is approximately 5.6 hours, and about 30% of NAC is renally excreted. NAC is available as an oral solution as well as
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i.v. and inhaled preparations. In concern to side effects, oral or inhaled NAC cause drowsiness, clamminess, stomatitis, hemoptysis, and rhinorrhea. According to the FDA, NAC is a category B pregnancy risk (Sansone and Sansone, 2011).

Uses of NAC:

Clinically NAC protects against paracetamol hepatotoxicity (Davreux et al., 1997). It also inhibits inflammation (Louwere et al., 1995), prevents apoptosis (Ferrari et al., 1995) and acts as a mucolytic agent (Dekhuijzen, 2004). Several studies indicate that pre- or post-treatment of NAC has neuro-protective properties (Carroll et al., 1998). Since NAC has anti-inflammatory properties (Blackwell et al., 1996) so it is used in collagen-induced arthritis (Tsuji et al., 1999) and in pulmonary edema caused by LPS administration (Gatti et al., 1993). As a thiol-containing compound, NAC has many protective effects in experimental endotoxemia and sepsis models. In early clinical septic shock, NAC decreases lipoperoxidative damage (Ortolani et al., 2000). NAC has a wide range of cardioprotective effects by scavenging approximately all pro-oxidative markers and potentiating antioxidant ability of cardiomyocytes in both pre- and post-ischemic insults resulting in restoration of cardio-dynamic parameters (Rosic et al., 2015). Regarding cardioprotection, NAC has a potent cardioprotective effect against cardiac changes induced by isoproterenol by decreasing lipoperoxidase and iso-prostane levels in the heart tissue and preventing free radicals-induced injury to the myocardium (Haleagrahara et al., 2011). Treatment with NAC during the first hours of severe clinical sepsis and septic shock decreases oxidative stress (Ortolani et al., 2000), enhances tissue oxygenation and cardiac output (Spies et al., 1998) and improves hepatic and respiratory functions (Rank et al., 2000). While delayed administration fails to enhance tissue oxygenation (Agusti et al., 2008).
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1997) and adversely affects survival rate in critically ill patients with established organ failure (Molnar et al., 1999). Infusion of a high dose of NAC over a short period results in a significant increase in cardiac output associated with a systemic vasodilatation (Rank et al., 2000) but these changes appear to be short lived and occur after administration of 150 mg/kg of NAC as a bolus dose (Walsh et al., 1999). Moreover, NAC slows the cardiac damage progression in the hypertensive rat model due to its capacity to restore glutathione status in cardiac tissue (Sochman, 2002). It also reduces MMP-2 gelatinolytic activity via direct inhibition (Bogani et al., 2007).

2- TAK 242 (ethyl-(6R)-[N-(2-chloro-4-fluorophenyl] sulfamoyl] cyclohex-1-hene-1-carboxylate)

TAK 242, or ethyl-(6R)-[N-(2-chloro-4-fluorophenyl] sulfamoyl] cyclohex-1-hene-1-carboxylate is a small molecule developed to inhibit the production of inflammatory mediators. It is a cell-permeable, TLR-4 signaling pathway inhibitor that binds specifically to TLR-4 with no binding affinity to other TLRs (Kawamoto et al., 2008; Matsunaga et al., 2011). Initially, it acts by decreasing the production of TNF-α, NO, IL-6 and IL-1β (Yamada et al., 2005). TAK 242 is able to inhibit TNF-α, IL-1β and IL-6 production in mice, although when the levels of these cytokine are already high while TAK 242 is administered (Takashima et al., 2009).

Figure (5): TAK 242 structure
**Pharmacodynamics:**

Mechanistically, TAK 242 binds selectively to a specific amino acid (Cys<sup>747</sup>) in the TIR domain of TLR-4 and subsequently disrupts the ability of TLR-4 to attach with adaptor proteins that are important for signal transduction (*Matsunaga et al.*, 2011) and inhibits both MyD88-dependent and MyD88 independent signaling pathway activated by LPS (*Takashima et al.*, 2009). TAK 242 has the ability to inhibit intracellular signaling pathway, with reduced phosphorylation of MAP kinases with no interference with the binding of LPS to TLR-4 (*Ii et al.*, 2006).

**Pharmacokinetics**

In-vitro pharmacokinetic studies show that TAK 242 is metabolized, by the cleavage of the sulfonamide bond, in to a phenyl ring moiety and a cyclohexene ring moiety. The phenyl ring moiety of TAK 242 produces M-1, 2-chloro-4-fluoroaniline, and M-1 is acetylated to form M-2 and conjugated to form the glucuronide (M-1-G). In addition, M-1 is converted to M-3 and M-4 by hydroxylation and subsequent sulfate conjugation. While the cyclohexene ring moiety of TAK 242 is metabolized to the glutathione conjugate, followed by further metabolism to form the cysteine conjugate and the mercapturic acid conjugate (*Jinno et al.*, 2012) as shown in the Figure (6). The pharmacokinetics of TAK 242 depends on the species and organs for example, it differs in dogs and rats also the distribution of TAK 242 is different in plasma and kidney (*Fumihiro et al.*, 2011; *Jinno et al.*, 2012). Furthermore, its concentration in plasma increases 3 hours after treatment and decreases at 24 hours after treatment (*Hua et al.*, 2015).
Experimental studies on TAK 242:

In a mouse model of endotoxemia, TAK 242 inhibits the pro-inflammatory response and decreases lethality in a dose-dependent manner (Sha et al., 2007). Importantly, similar benefits can be obtained if treatment is giving up to 2 hours after the LPS challenge. Use of TAK 242 in endotoxemia model using conscious guinea pig shows better control of hemodynamic parameter, decreased high mobility group box-1 (HMGB-1) level and a dose-dependent improved survival (Kuno et al., 2009). In addition, administration of TAK 242 to mice 1 h before LPS administration causes dose-dependent inhibition of TNF-α, IL-1β, IL-6, IL-10.
and NO metabolites. Also it causes dose-dependent protection of mice from lethality. Interestingly, TAK 242 shows benefits even when given after LPS administration (Sha et al., 2007). In a mouse model of CLP, TAK 242 increases survival rates from 17 to 50% and improves organ injury when given together with antibiotics (Sha et al., 2011).
1.8 Hypothesis

In this study, we hypothesize that TLR-4 induces myocardial injury through up regulation of MMP-2 following experimentally induced poly-microbial sepsis by cecal ligation and puncture model.

1.9 Aims of the study

1. To examine the effectiveness of NAC and TAK 242 in improving myocardial injury following sepsis.
2. To study the role of NAC and TAK 242 in modulation of proinflammatory mediators.
3. To investigate the signaling pathway of MMP-2 in mechanistic action of NAC and TAK 242.
Chapter Two

Materials & Methods
2. Materials

The drugs, chemicals, and instruments that are used in this study with their supplier are listed in tables (1) and (2).

Table (1): Instruments and Their Supplier

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Elisa Reader</td>
<td>Bio-Tek Instruments. Inc., USA</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Hettich, Germany</td>
</tr>
<tr>
<td>Fine Surgical Tool</td>
<td>Biotechno, Germany</td>
</tr>
<tr>
<td>High Intensity Ultrasonic Liquid Processor</td>
<td>Sonics &amp; Materials Inc., USA</td>
</tr>
<tr>
<td>Sensitive Electrical Balance</td>
<td>Denver, Germany</td>
</tr>
<tr>
<td>Surgical Sutures</td>
<td>Ethicon, Norderstedt, Germany</td>
</tr>
<tr>
<td>Vortex mixer</td>
<td>Cypress diagnostics, Belgium</td>
</tr>
</tbody>
</table>
Table (2): List of Chemicals, Pharmaceuticals, Reagents and their supplier.

<table>
<thead>
<tr>
<th>Pharmaceuticals and Reagents</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamine</td>
<td>DeltaSelect, Dreieich, Germany</td>
</tr>
<tr>
<td>Mouse Cardiac troponin I ELISA kit</td>
<td>Cloud and clone, USA</td>
</tr>
<tr>
<td>Mouse TNF-α ELISA kit</td>
<td>Bosterbio, USA</td>
</tr>
<tr>
<td>Mouse MCP1 ELISA kit</td>
<td>Bosterbio, USA</td>
</tr>
<tr>
<td>Mouse IL-1β ELISA kit</td>
<td>Bosterbio, USA</td>
</tr>
<tr>
<td>Mouse IL-6 ELISA kit</td>
<td>Bosterbio, USA</td>
</tr>
<tr>
<td>N-acetyl cysteine</td>
<td>Santa Cruz Biotechnology, USA</td>
</tr>
<tr>
<td>RNA PCR kit</td>
<td>Applied Biosystems, Foster City, CA, USA</td>
</tr>
<tr>
<td>Trizol-Reagent</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>Triton-x-100</td>
<td>Promega, Wisconsin, USA</td>
</tr>
<tr>
<td>TAK 242</td>
<td>Med Chem Express, USA</td>
</tr>
<tr>
<td>Xylazine</td>
<td>Bayer, Leverkusen, Germany</td>
</tr>
</tbody>
</table>
2.1 Preparation of Animals

A total of sixty Adult male Albino-Webster mice were used in this study. Their weights ranged from 30 to 35 g. The study was conducted at Kufa University, College of Medicine, Department of Pharmacology and Therapeutics from November 2015 to April 2016. Mice were kept in the animal house of the institute, in a temperature-controlled room on a 12:12-h light-dark cycle with free access to water and regular chow diet. The experimental protocols and procedures was approved by the local ethical committee, and all animals were treated and handled according to the Guide for Care and Use of Laboratory Animals of the National Research Council as well as institutional guidelines.

2.2 Induction of Poly-microbial Sepsis in Mice

Cecal ligation and puncture (CLP) were performed to induce sepsis according to the original CLP protocol introduced by (Wichterman et al., 1980). Firstly, mice were harvested overnight except for free water before CLP. Then, they were anesthetized with 1.5 ml/kg of a ketamine (100 mg/ml): xylazine (20 mg/ml) solution in a 2:1 ratio and they were immobilized on an aseptic operating table (Khan et al., 2013). In a sterile operation environment, a 2 cm abdominal midline incision was made to expose the cecum, which was ligated below the ileocecal valve and punctured twice with an 18-gauge needle. A small amount of stool was squeezed through the puncture site. The bowel was then situated back in the abdomen and the incision was sutured with a sterile 5-0 silk (Maitra et al., 2010). Immediately after surgery, a single dose of lactated Ringer solution; 50 mL/kg of body weight) was administered by s.c. for resuscitation. Mice were placed back in their cages. Mice were monitored for various signs of sickness for 24 hour. The sham group underwent a sham surgery in which the abdominal cavity was opened
to find the cecum and the abdominal wall incision was sutured, but neither ligating nor puncturing was done.

**2.3 Study Design**

After adaptation period of 2 weeks, the animals were randomly divided into 5 groups (12 mice each) as following:

1. **Sham surgical operated group (Sham):** all mice of this group were subjected to sham surgery and served as the surgical control group.

2. **Cecal ligation and puncture operated group (CLP):** all mice of this group were subjected to cecal ligation puncture surgery (CLP) and served as sepsis control group.

3. **Vehicle pre-treated group:** all mice of this group were given 10ml/kg of DMSO (5%) i.p. 1 hour before they were subjected to cecal ligation puncture surgery (CLP).

4. **N- Acetylcysteine pre-treated group (NAC):** all mice of this group were treated i.p. with 150 mg/Kg of NAC 1 hour before they were subjected to cecal ligation puncture surgery (CLP).

5. **TAK-242 pre-treated group:** all mice of this group were treated i.p. with 3mg/Kg of TAK-242 1 hour before they were subjected to cecal ligation puncture surgery (CLP).
2.4 Preparation of drugs and chemicals:

2.4.1 N-acetylcysteine (NAC)

N-acetylcysteine was used in a dose 150mg/Kg according to the previous studies (Ozdulger et al., 2003; Glantzounis, 2004). It was dissolved in DMSO and given i.p. to the animal according to the body weight 1 hour before CLP (Rocksén, 2003).

2.4.2 TAK 242

TAK 242 1-cyclohexene-1-carboxylic acid, 6-[[2-chloro-4 fluorophenyl]amino]sulfonyl]-, ethyl ester (TAK 242) was purchased from Med Chem Express Company. TAK 242 was administered at a dose of 3mg/kg according to the previous studies (Kuno et al., 2009; Hua et al., 2015). It was dissolved in DMSO and given i.p. to the animal according to the body weight 1 hour before CLP (Sha et al., 2007; Kuno et al., 2009).

2.5. Outcome measurements

1. ELISA analysis for determination of:
   a. Inflammatory markers: pro-inflammatory cytokines and chemokine (TNF-α, IL-1β, IL-6 and MCP1).
   b. Marker for cardiac injury: cTn-I

2. Real time PCR for MMP-2 quantification
   To quantify MMP-2 that was expressed.
Materials and Methods

Chapter Two

3. Histology of cardiac tissue

The basal side of the heart was fixed in 10% formalin, then processed by routine histological methods, sectioned in a standard fashion and stained with H&E. Slides assessed in a blinded fashion by a pathologist and scored.

4. Cardiac function measurements

To measure the left ventricle functions which include: heart rate, ejection fraction % (EF %), left ventricular end systolic pressure (LVESP), and left ventricular end diastolic pressure (LVEDP), cardiac output (CO).

2.6 Cardiac function measurements

Twenty four hours after cecal ligation and puncture procedure, cardiac function was assessed as described previously (Yousif and Al-amran, 2011; Slimani et al., 2014). Briefly, Mice were anesthetized with 1.5 ml/kg of a ketamine (100 mg/ml)/xylazine (20 mg/ml) solution in a 2:1 ratio (Khan et al., 2013). Animals were laid supine on a heating blanket and body temperature was maintained at range 37°C ± 0.5°C. The external right carotid artery was exposed, and a micro-tipped transducer catheter (1.4F, Millar Instrument Inc.) was placed into the artery and then advanced into the left ventricle. The other end of the catheter was connected to an electrostatic chart recorder (model ES 2000, Gould, Cleveland, USA) and Pressure-volume loops recorded to measure the maximum rate of change in ventricular pressure and ejection fraction by using the MPVS-400 system with the aid of P van software (Conductance Technologies, San Antonio, TX, and Millar, Houston, TX) was used to measure all data which include: Heart rates (HR), left ventricle end-diastolic pressure (LVEDP), left ventricle end-systolic pressure (LVESP), EF%, cardiac output (CO).
2.7 Collection of Samples

After assessment of Cardiac function, Blood sample was drawn using direct needle puncture of the heart. For heart collection, a thoracic operation was performed; the heart of the mice was exteriorized and cut into two parts: the apical side of the heart was snap-frozen until to be used for preparation of the heart homogenate while the basal side of the heart was immediately fixed in 10% formalin, then processed by routine histological methods.

2.7.1 Preparation of Plasma Sample for TNF-α, IL-1β, IL-6, MCP-1 and cTn-I

Blood was placed in a tube containing heparin as anticoagulant and mixed thoroughly then centrifuged at 3000 rpm for 15 min. then stored at −20°C until used for determination of TNFα, IL-1β, IL-6, MCP-1 and cTn-I.

2.7.2 Preparation of Tissue Sample for TNF-α, IL-1β, IL-6 and MCP-1

Preparation of heart homogenate was done by weighing the apical side of the heart tissues (that had been rinsed with ice cold saline to remove any clots or RBCs) and homogenizing with (1:10w/v) of 0.1 M (PBS) phosphate buffered saline (PH 7.4) which contained protease inhibitor cocktail and 1% Triton-100 (Zhang et al., 2005).

The 10% homogenates were centrifuged at 2,500 g for 20 min at 4°C to obtain the supernatants which were used for determination of TNFα, IL-1β, IL6 and MCP-1 (Zhang et al., 2005).
2.7.3 Tissue Sampling for Histopathology

The basal side of the heart was put in 10% neutral buffered formalin and left for 72 hour for fixation. After well fixation, the specimens were dehydrated by passing them through a graded series of ethanol for two hour for each (70%, 80%, 90% and 100%) then the specimens were cleared for one hour in two changes of xylene after that the specimens was embedded in melted paraffin and allowed to harden. The blocks were sectioned at 5µm thickness by rotary microtome equipped with disposable steel knives. Sections are flattened on a water bath at 37°C, floated on to microscope slides and allowed to dry then stained with Mayer’s Hematoxylin and Eosin routine stain for identification of the pathological changes (Bancroft and Stevens, 2010).

After staining, evaluation of scores was done by a pathologist who was blinded to the experimental treatment groups. A scoring system (0, 1, 2, 3, and 4) was used to classify the heart tissue changes, myocardial necrosis, according to the severity of the damage (Zingarelli et al., 2002). According to this score system the following criteria were used: score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling; score 3 (severe), the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), the presence of contraction bands, leukocyte infiltrate, and hemorrhage.
2.8 ELISA for Determination of TNF-α, IL-1β, IL-6 and MCP-1

Commercial ELISA kits (Bosterbio, USA) were utilized to quantify MCP-1, TNF-α, IL-1β and IL-6 in plasma and myocardial tissue homogenate, while commercial ELISA kits (cloud & clone, USA) was utilized to quantify plasma cardiac Troponin-I (cTn-I). Samples and standards were prepared according to manufacturer's instructions. Absorbance of standards and samples were determined spectrophotometrically at 450 nm, by a microplate reader (Bio-Rad Laboratories, CA, USA). Obtained data were plotted against the linear portion of a standard curve (Turler et al., 2015).

2.9 Real-time PCR for Quantification of MMP-2

Total RNA was extracted by using Trizol-Reagent (Invitrogen, Carlsbad, CA) and equal amounts (1μg) of RNA were reverse transcribed using a RNA PCR kit (Applied Biosystems, Foster City, CA, USA) as described previously (Wang et al., 2006). We used Primer Express software (Applied Biosystems) for mice gene PCR primer sequences and amplicon sizes for real-time PCR, the Primer sequences as follow:

MMP-2 Primer sequences
Sense 5′ -CAAGTTCCCCGGCGATGTC-3′
Antisense 5′ - CTGGTCAAGGTCACCTGTC-3′

B-actin Primer sequences
Sense 5′ -AGAGAGAGGCCCTCACGTTGCT-3′
Antisense 5′ -TTGTGCGGGAGATGCTCAGTG-3′
2.10 Statistical Analysis

Statistical analysis data was performed using Stat View software (Abacus Concepts, USA). Analysis of variance (ANOVA) with Fisher post-hoc test was used to investigate differences between mice, and data differences were confirmed using the Mann-Whitney U-test. Statistically the present data significance was defined as $P \leq 0.05$. 
Chapter Three

Results
3.1 Effect of Sepsis on Inflammatory Markers

3.1.1 Effect of Sepsis on Plasma Pro-Inflammatory Cytokines (TNF-α, IL-1β and IL-6)

At the end of the experiment (24 hours after cecal ligation and puncture), the levels of plasma pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) were significantly ($p<0.05$) increased in CLP group as compared with sham group. There was insignificant difference ($p>0.05$) between vehicle and CLP group. The plasma pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) levels of both NAC and TAK 242 pre-treated groups were significantly ($p<0.05$) lower than that of CLP and vehicle groups while their levels in the NAC group was significantly ($p<0.05$) lower than that of TAK 242 group. The changes in plasma pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) levels are summarized in Figure (7).
Figure (7): The mean values of plasma pro-inflammatory cytokines (pg/ml) in the five experimental groups 24 hours after cecal ligation and puncture.

Data are expressed as mean±standard error, n = 8 in each group; *p <0.05 CLP and vehicle groups versus corresponding sham group; **p <0.05 NAC and TAK 242 groups versus CLP mice; ***p <0.05 TAK 242 group versus NAC group.
3.1.2 Effect of Sepsis on Myocardial Pro-inflammatory Cytokines (TNF-α, IL-1β and IL-6)

At the end of the experiment (24 hours after cecal ligation and puncture), the levels of myocardial pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) were significantly (p<0.05) increased in CLP group as compared with sham group. There was insignificant difference (p>0.05) between vehicle and CLP group. The myocardial pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) levels of both NAC and TAK 242 pre-treated groups were significantly (p<0.05) lower than that of CLP and vehicle groups while their levels in the NAC group was significantly (p< 0.05) lower than that of TAK 242 group. The changes in myocardial pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) levels are summarized in Figure (8).
Figure (8): The mean values of myocardial pro-inflammatory cytokines (pg/mg) in the five experimental groups 24 hours after cecal ligation and puncture.

Data are expressed as mean ± standard error, n = 8 in each group; *p <0.05 CLP and vehicle groups versus corresponding sham group; **p <0.05 NAC and TAK 242 groups versus CLP mice; ***p <0.05 TAK 242 group versus NAC group.
3.2 Effect of Sepsis on plasma and myocardial MCP-1

The plasma and myocardial MCP-1 levels were significantly (p<0.05) increased in CLP and group as compared with sham group. There was insignificant difference (p>0.05) between vehicle and CLP group. The plasma and myocardial MCP-1 level of both NAC and TAK 242 pre-treated groups was significantly (p<0.05) lower than that of CLP and vehicle groups and its level in the NAC group was significantly (p<0.05) lower than that of TAK 242 group. The changes in plasma and myocardial MCP-1 are summarized in Figure (9, 10).

Figure (9): The mean values of plasma MCP-1 level (ng/ml) in the five experimental groups 24 hours after cecal ligation and puncture.

Data are expressed as mean±standard error, n = 8 in each group; *p <0.05 CLP and vehicle groups versus corresponding sham group; **p <0.05 NAC and TAK 242 groups versus CLP mice; ***p <0.05 TAK 242 group versus NAC group.
Figure (10): The mean values of myocardial MCP-1 level (ng/mg) in the five experimental groups 24 hours after cecal ligation and puncture.

Data are expressed as mean ± standard error, n = 8 in each group: * p <0.05 CLP and vehicle groups versus corresponding sham group; ** p <0.05 NAC and TAK 242 groups versus CLP mice; *** p <0.05 TAK 242 group versus NAC group.
3.3 Effect of Sepsis on Matrix Metalloproteinase-2 (MMP-2) expression in the myocardial tissue

Myocardial tissue homogenates with total RNA was extracted, and RT-PCR experiments were performed using primers recognizing MMP-2. The MMP-2 expression in myocardial cells was significantly (p<0.05) increased in CLP and vehicle groups as compared with sham group. There was insignificant difference (p>0.05) between vehicle and CLP group. The MMP-2 level of NAC and TAK 242 pre-treated groups was significantly (p<0.05) lower than that of CLP group. The MMP-2 level of NAC group was significantly (p<0.05) lower than that of TAK 242 group. The changes in MMP-2 level are summarized in Figure (11).
Figure (11): The mean values of relative MMP2 activity in the five experimental groups 24 hours after cecal ligation and puncture.

Data are expressed as mean ± standard error, n = 8 in each group; *p <0.05 CLP and vehicle groups versus corresponding sham group; **p <0.05 NAC and TAK 242 groups versus CLP mice; ***p <0.05 TAK 242 group versus NAC group.
3.4 Effect of Sepsis on Plasma cTn-I

The plasma level of the cardiac injury marker (cTn-I) was significantly (p<0.05) increased in CLP and vehicle groups as compared with sham group. There was insignificant difference (p>0.05) between vehicle and CLP group. The cTn-I level of NAC and TAK 242 pre-treated groups was significantly (p<0.05) lower than that of CLP group. The cTn-I level of NAC group was significantly (p<0.05) lower than that of TAK 242 group. The changes in cTn-I level are summarized in Figure (12).

Figure (12): The mean values of plasma cTn-I (pg/ml) in the five experimental groups 24 hours after cecal ligation and puncture.

Data are expressed as mean ± standard error, n = 8 in each group; * p <0.05 CLP and vehicle groups versus corresponding sham group; ** p <0.05 NAC and TAK 242 groups versus CLP mice; *** p <0.05 TAK 242 group versus NAC group.
3.5 Histopathological Finding

Myocardial injury was assessed in the mouse's heart of the five experimental groups. To semi-quantify the difference in cardiac damage, histological sections from all groups were examined and scored according to the protocol of Zingarelli (Zingarelli et al., 2002). Eight animals in each group were included, and five sections from each animal were evaluated at the end of the study and the results are as follow:

3.5.1 Sham group

A cross section of sham mouse s' heart showed the normal cardiac structure: no interstitial edema and focal necrosis, no diffuse myocardial cell swelling and necrosis; no contraction bands, no neutrophil infiltration, no capillaries compressing and no hemorrhage. All mice in this group showed normal heart 100% as shown in Table (3) & Figure (13A).

3.5.2 CLP group

Histologically, myocardial tissue from CLP group 24 hours after induction of sepsis revealed a marked myocardial injury with the development of contraction bands and polymorphonuclear leukocytes infiltration besides interstitial edema and localized extravasation of red blood cells. There was a statistically significant difference between CLP group and sham group (p <0.05) and the total severity scores of the CLP group showed that 87.5% of the group had severe cardiac injury and 12.5% had highly severe cardiac injury as shown in Table (3) & Figure (13B).
3.5.3 Vehicle group

There was statistically insignificant difference between vehicle group and CLP group (p>0.05) and the total severity scores of the vehicle group showed that 87.5% of the group had severe cardiac injury and 12.5% had highly severe cardiac injury as shown in Table (3) & Figure (13C).

3.5.4 NAC group

Treatment of mice with NAC improved cardiac injury significantly (p <0.05) as compared with CLP and vehicle groups and the total severity score of this group showed that 87.5% had mild cardiac injury and 12.5% had moderate cardiac injury Table (3) & Figure (13D).

3.5.5 TAK 242 group

Treatment of mice with TAK 242 improved cardiac injury significantly (p <0.05) as compared with CLP and vehicle groups and the total severity score of this group showed that 25% had mild cardiac injury and 75% had moderate cardiac injury Table (3) & Figure (13E).
Table (3): Difference in the histopathological grading of abnormal cardiac changes between the 5 experimental groups.

<table>
<thead>
<tr>
<th>Histopathological grading</th>
<th>Study groups</th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Vehicle</td>
<td>CLP</td>
<td>NAC</td>
<td>TAK 242</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
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<tr>
<td>No abnormality (Score 0)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Mild (Score 1)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Moderate (Score 2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Severe (Score 3)</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>87.5</td>
<td>7</td>
<td>87.5</td>
<td>0</td>
</tr>
<tr>
<td>Highly severe (Score 4)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>12.5</td>
<td>1</td>
<td>12.5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>100</td>
<td>8</td>
<td>100</td>
<td>8</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>Median</td>
<td>No abnormality</td>
<td>Severe</td>
<td>Severe</td>
<td>Mild</td>
<td>Moderate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The Results

Chapter Three

Figure (13): NAC and TAK 242 treatment improved histopathological changes after sepsis. Heart tissue from CLP demonstrating extensive contraction band change (black arrows) and extensive extravasation of red blood cells (white arrowhead) with margination of poly-morphonuclear leukocytes (white arrows) and interstitial edema (black arrowhead) (Fig. 11 A, B, C, D, E, F).
3.5.6 Changes in Total severity score of the study groups

At the end of the experiment, the level of cardiac injury was significantly (p<0.05) increased in CLP and vehicle groups as compared with sham group (3.5±0.11; *p<0.05). Zingarelli system indicates that the damage score was significantly reduced in NAC (1±0.13; **p<0.05) and TAK 242 (2.5±0.31; **#p<0.05) treated mice as compared with the CLP mice 24 hours after induction of sepsis. Levels of cardiac injury are shown in Figure (14).

**Figure (14): The difference in mean values of total severity scores in the 5 experimental groups.**

Data are expressed as mean±standard error, n = 8 in each group; *p <0.05 CLP and vehicle groups versus corresponding sham group; **p <0.05 NAC and TAK 242 groups versus CLP mice; ***p <0.05 TAK 242 group versus NAC group.
3.6 Effect of Sepsis on LV Function

To investigate the effect of pre-treatment with NAC and TAK 242 on the sepsis induced myocardial dysfunction, LV function was assessed at 24 hours after cecal ligation and puncture. As shown in Table (4) CLP group has significantly (p<0.05) decreased level of ejection fraction, cardiac output and LVESPV; while it has significantly (p<0.05) elevated level of heart rate and LVEDP as compared with sham group. There was insignificant difference (p>0.05) between vehicle and CLP group, while NAC and TAK 242 pretreated groups show significantly (p<0.05) elevated level of ejection fraction, cardiac output and LVESPV; while they have significantly (p<0.05) diminished level of heart rate and LVEDP indicating that NAC and TAK 242 pre-treatment attenuate sepsis induced myocardial dysfunction.
Table (4): The mean values of left ventricular function parameters of the five experimental groups 24 hour after cecal ligation and puncture.

Data are expressed as mean ± standard error, \( n = 8 \) in each group; * \( p < 0.05 \) CLP and vehicle groups versus corresponding sham group; ** \( p < 0.05 \) NAC and TAK 242 groups versus CLP mice; *** \( p < 0.05 \) TAK 242 group versus NAC group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heart rate (bpm)</th>
<th>LVEDP (mmHg)</th>
<th>Ejection fraction (%)</th>
<th>LVESP (mmHg)</th>
<th>Cardiac Output (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>422 ± 14</td>
<td>3.3±1.2</td>
<td>63.1 ± 2</td>
<td>121.1±1.2</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>485 ± 12*</td>
<td>8.1±1.4*</td>
<td>32.5 ± 1</td>
<td>53.1±1.4*</td>
<td>4.2 ± 1.4*</td>
</tr>
<tr>
<td>CLP</td>
<td>478 ± 21*</td>
<td>7.6±1.5*</td>
<td>32.5 ± 1.5</td>
<td>51.5±1.2*</td>
<td>3.0 ± 1.4*</td>
</tr>
<tr>
<td>NAC</td>
<td>462 ± 12**</td>
<td>3.8±1.4**</td>
<td>55.4 ± 1**</td>
<td>99.3±1.2**</td>
<td>4.9 ± 1.4**</td>
</tr>
<tr>
<td>TAK 242</td>
<td>471 ± 3**#</td>
<td>5.1±1.2**#</td>
<td>41.1 ± 1.2**#</td>
<td>84.5±2.2**#</td>
<td>3.9 ± 1.6**#</td>
</tr>
</tbody>
</table>
3.7 Correlation between ejection fraction and different markers.

3.7.1 Correlation between ejection fraction and TNF-α level

The mean decrease in EF% value showed a significant association with increased TNF-α level. There was a strong negative correlation with plasma and myocardial TNF-α levels (r = -0.908, p < 0.05, r = -0.911, p < 0.05 respectively) as shown in table (5) & figure (15, 16) respectively.

3.7.2 Correlation between ejection fraction and IL-1β level

The mean decrease in EF% value showed a significant association with increased IL-1β level. There was a strong negative correlation with plasma and myocardial IL-1β levels (r = -0.939, p < 0.05, r = -0.906, p < 0.05) as shown in table (5) & figure (17, 18) respectively.

3.7.3 Correlation between ejection fraction and IL-6 level

The mean decrease in EF% value had a significant association with increased IL-6 level. There was a strong negative correlation with plasma and myocardial IL-6 levels (r = -0.911, p < 0.05, r = -0.898, p < 0.05) as shown in table (5) & figure (19, 20) respectively.

3.7.4 Correlation between ejection fraction and MCP-1 level

The mean decrease in EF% value was significantly associated with increased MCP-1 level. There was a strong negative correlation with plasma and myocardial MCP-1 levels (r = -0.977, p < 0.05, r = -0.946, p < 0.05) as shown in table (5) & figure (21, 22) respectively.
3.7.5 Correlation between ejection fraction and cTn-I level

The mean decrease in EF% value had a significant association with increased plasma cTn-I level. There was a strong negative correlation with cTn-I \( (r = -0.953, p < 0.05) \) as shown in table (5) & figure (23).

3.7.6 Correlation between ejection fraction and Relative MMP-2 Activity

The mean decrease in EF% value significantly associated with increased MMP-2 activity. There was a strong negative correlation with MMP-2 activity. \( (r = -0.972, p < 0.05) \) as shown in table (5) & figure (24).
Table (5): Correlation between ejection fraction and different parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Correlation Value</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF% &amp; plasma TNF-α</td>
<td>-0.908</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; myocardial TNF-α</td>
<td>-0.911</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; plasma IL-1β</td>
<td>-0.939</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; myocardial IL-1β</td>
<td>-0.906</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; plasma IL-6</td>
<td>-0.911</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; myocardial IL-6</td>
<td>-0.898</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; plasma MCP-1</td>
<td>-0.977</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; myocardial MCP-1</td>
<td>-0.946</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; plasma cTn-I</td>
<td>-0.953</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>EF% &amp; relative MMP-2 activity</td>
<td>-0.972</td>
<td>P&lt; 0.05</td>
</tr>
</tbody>
</table>

Figure (15): Correlation between plasma TNF-α and ejection fraction. Note the strong negative correlation between two parameters.
Figure (16): Correlation between myocardial TNF-α and ejection fraction. Note the strong negative correlation between two parameters.

Figure (17): Correlation between plasma IL-1β and ejection fraction. Note the strong negative correlation between two parameters.
Figure (18): Correlation between myocardial IL-1β and ejection fraction. Note the strong negative correlation between two parameters.

Figure (19): Correlation between plasma IL-6 and ejection fraction. Note the strong negative correlation between two parameters.
Figure (20): Correlation between myocardial IL-6 and ejection fraction. Note the strong negative correlation between two parameters.

Figure (21): Correlation between plasma MCP-1 and ejection fraction. Note the strong negative correlation between two parameters.
Figure (22): Correlation between myocardial MCP-1 and ejection fraction. Note the strong negative correlation between two parameters.

Figure (23): Correlation between plasma cTn-I and ejection fraction. Note the strong negative correlation between two parameters.
Figure (24): Correlation between relative MMP-2 activity and ejection fraction. Note the strong negative correlation between two parameters.
Chapter Four

Discussion
4. Discussion

Myocardial injury is a major consequence of septic shock which results in the high mortality rate among sepsis patients in the intensive care units (Bulmer, 2011). To understand the pathway of sepsis induced myocardial injury through down regulation of MMP-2 pathway, the present study has used NAC and TAK 242 to investigate their cardio-protective potential. In this study, mice model of CLP is challenged to produce sepsis which is regarded as the cornerstone for modeling of poly-microbial sepsis. This model has initial benefits because it produces the dynamic changes in cardiovascular function that occurs in humans with sepsis. Additionally, CLP causes the massive release of proinflammatory cytokines (Buras et al., 2005). Furthermore; CLP model has several aspects that address the complicated clinical course of sepsis in human: these include tissue trauma due to a laparotomy, necrosis caused by a cecum ligation, and infection as a result of the peritoneal microbial flora leakage into the peritoneum. The latter process finally results in septic shock due to inflammatory response activation which results in the translocation of enteric bacteria into the bloodstream (Dejager et al., 2011). So polymicrobial sepsis is characterized by an early hyperdynamic phase followed by a late hypodynamic phase which is similar to the human (Rittirsch et al., 2009).
4.1 Effects of Sepsis on Study Parameters

4.1.1 Effect of Sepsis on Pro-Inflammatory Cytokines and Chemokines (TNF-α, IL-1β, IL-6 and MCP-1)

A number of published reports have investigated and confirmed that myocardial injury during sepsis is related with inflammatory mediator’s expression, including TNF-α, IL-1β and IL-6 (Saito et al., 2010; Lohner et al., 2013). Furthermore, pro-inflammatory cytokines have been up-regulated after acute injury caused by sepsis which directly attenuate cardiac contractility and induce myocardial apoptosis that contribute, in some part, to the mechanism of exaggerated cardiac depression in experimental sepsis mice model (Cha et al., 2008; Wondergem et al., 2010). Additionally, administration of TNF-α or IL-1β directly depress the contractility in cultured cardiomyocytes (Wondergem et al., 2010), and this adverse effects of pro-inflammatory cytokines can be ameliorated by antibodies that show a rapid improvement in cardiac contractility (Bujak and Frangogiannis, 2009). In this study, we demonstrate that sepsis enhanced the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in both plasma and cardiac tissue of CLP and vehicle groups as compared with sham group, which associated with worse LV function. This finding is in agreement with that reported by (Lohner et al., 2013; Zhou et al., 2016). The enhanced plasma cytokines level can be explained by the notion that stimulation of neutrophils, macrophages, monocytes and endothelial cells possibly occurs through TLR-4 that initiates downstream signaling pathway and results in the activation of NF-κB, which leads to the transcription of a great number of pro-inflammatory cytokines (Feng and Chao, 2011). It is noteworthy that cardiomyocytes are able to produce TNF-α, IL-1β, IL-6 possibly through TLR-4 which may explain the increased cytokines level in the myocardium in
addition to pro-inflammatory cytokines produced by macrophages, infiltrated neutrophils and fibroblast (Carlson et al., 2005).

4.1.2 Effect of Sepsis on Monocyte Chemo-attractant Protein (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) is one member of the chemokine family, expressed by many cell types and is responsible for up-regulation of infiltration and migration of monocytes and neutrophils (Renzo et al., 2015). In the present data, we investigated that MCP-1 levels in plasma and cardiac tissue are significantly higher in the CLP and vehicle than sham mice, which is associated with extensive contraction band change, extensive extravasation of red blood cells with margination of poly-morphonuclear leukocytes (PMN) and interstitial edema. This finding is confirmed by (Labbe et al., 2010; Slimani et al., 2014) and possibly either due to its constitutive expression or due to stimulatory agents such as TNF-α that regulate MCP-1 expression at the transcriptional level (Melgarejo et al., 2009).
4.1.3 Effect of Sepsis on Matrix Metalloproteinase (MMP-2) Expression in the Myocardial Tissue

The collagen network is a crucial component of the heart’s extracellular matrix (ECM) where it works to secure and support cardiomyocytes in proper alignment required for coordinated contraction and it helps in determining heart compliance. The ECM is mainly composed of collagen with smaller proportion contributed by elastin, laminin and fibronectin. Cardiac extracellular matrix modulation occurs by many members of the large matrix metalloproteinase (MMP) family. Interestingly, MMP-2 (also known as gelatinase A) contributes to the cardiovascular injury and remodeling in many diseases due to its ability to degrade extra- and intracellular proteins (Chow et al., 2007; Raffetto and Khalil, 2008). So inhibition of MMP-2 activity with non-selective agents is associated with the improvement of left ventricular injury and modulation of chronic heart failure models (Sakata et al., 2004; Chen et al., 2004). Our study demonstrated that the expression of MMP-2 is up-regulated or it is stably expressed in CLP and vehicle groups as compared with sham mice. This finding is also evidenced by (Wohlschlaege et al., 2005; Cena et al., 2012). In contrast to (Lalu et al., 2004), observed that MMP-2 activity deregulated with loss of MMP-2 protein after the induction of sepsis. Our data suggest that MMP-2 activity is highly regulated at the transcriptional level and its expression can be up-regulated in cardiac cells in response to increased production of pro-inflammatory cytokines as observed by (Gao et al., 2003; Bergman et al., 2003). Consequently, MMP-2 activation results in proteolysis of troponin-I, myocin light chain-1, α-actinin and titin which are essential proteins for cardiomyocytes contractility thereby results in decreased myocardial contractility (Schulz, 2007).
4.1.4 Effect of Sepsis on Cardiac Injury Marker

In this study, sepsis unfavorably increases the plasma level of cTn-I in CLP and vehicle groups as compared with sham group. This result is in agreement with that reported by (Zhang et al., 2015; Abdelrahman et al., 2015; Lu et al., 2015). cTn-I is a cardiac injury marker that has been used to reflect the degree of myocardial injury after sepsis with high sensitivity and specificity (Willott et al., 2010). Thus the increase of cTn-I level may reflect the underlying myocardial injury since it is increased only when there is myocardial injury (Wu et al., 2001). Therefore the observed elevation may indicate that there is myocardial damage when sepsis occurs; the myocardial cells are damaged, the membrane permeability is increased and troponin moves out of the cells, thus elevating levels of troponin in the blood (Roongsritong et al., 2004; Van Bockel et al., 2005).

4.1.5 Effect of Sepsis on Histological Changes in the Heart

In this study, histological examination of mouse hearts is observed under a light microscope (x40 and x100 magnification). In the sham group, clear myocardial structures are visible and regularly arranged cardiac muscle fibers are observed with clear cross striations and normal structures. On the other hand, heart from CLP and vehicle groups reveal a marked myocardial injury with the development of contraction bands and polymorphonuclear leukocytes infiltration besides interstitial edema, myocardial necrosis and localized extravasation of red blood cells. Similar finding were reported by (Lu et al., 2015; Zhang et al., 2015; Tomita et al., 2015).

A reasonable explanation for these structural changes of the heart is that: during sepsis the endothelium is activated and this will lead to attraction of inflammatory...
cells that invade the myocardium. This leads to edema which can result in decreased myocardial compliance, but may also impair regional blood flow that will result in cytopathic hypoxia and subsequent mitochondrial damage. Moreover, activation of apoptotic pathways by proinflammatory mediators may eventually result in disruption of the actin/myosin contractile apparatus and loss of integrity of cardiomyocytes detectable by increased plasma cTn-I (Fenton et al., 2004). Likewise, activation of MMP-2 results in myofibrillar disruption and decreases cardiac contractility and may contribute to sepsis-induced myocardial injury (Gao et al., 2003).

4.1.6 Effect of Sepsis on Left Ventricular Function

Since left ventricular ejection fraction is a strong indicator and the most significant index of heart function that is currently in clinical use (Lu and Mukkamala, 2006). So we rely on it more specifically to evaluate the effect of sepsis on the heart. In this study, sepsis results in worse Left ventricular function performance in CLP and vehicle groups as compared with sham group manifested as 32.5% reduction in EF. This observation is in agreement with studies of (Smeding et al., 2012; Li et al., 2013; Lohner et al., 2013). Taken together, disruption of contractility apparatus by pro-inflammatory mediators and MMP-2, myocardial edema induced left ventricle stiffness and loss of cardiomyocytes integrity as detected by increasing level of cTn-I which may be contributing factors, participating in impaired myocardial contractility which results in myocardial injury as detected by a drop in ejection fraction.
4.2 Effects of Drug Treatment

4.2.1 Effects of Pre-Treatment with NAC on Sepsis Induced Myocardial Injury

In this study, NAC is found to produce a marked reduction in both plasma and cardiac tissue levels of pro-inflammatory cytokines and chemokine (TNF-α, IL-1β, IL-6 and MCP-1). Similar findings are obtained by (Victor et al., 2003; Senoglu et al., 2008; Mukherjee et al., 2010). In contrary to (Ohnishi et al., 2014) suggested that long-time low-dose NAC treatment increases expressions of proinflammatory cytokines through enhancement of kinase phosphorylation while (Emet et al., 2004) found that NAC did not affect cytokine levels. The observed reduction in pro-inflammatory cytokines may be attributed to anti-inflammatory effect of NAC likely through inhibition of NF-κB (Berk et al., 2008).

Concerning MMP-2 expression, this study shows that treatment with NAC greatly down-regulated the MMP-2 expression in myocardial cells. This observation is also noticed by (Adubeiro et al., 2004). This finding suggests that anti-inflammatory effect of NAC in addition to the restoration of glutathione content in cardiac tissue which may be responsible for reduced expression of MMP-2 (Galis et al., 1998; Bourraindeloup et al., 2004).

Moreover, this study demonstrates that NAC administration improves cardiac injury as detected by meanwhile reduction in cTn-I. This finding is in agreement with (Koramaz et al., 2006; Liu et al., 2010; Mahmoud et al., 2011).

In the present study, it is noticed that the severity of cardiac injury is significantly improved by NAC administration. This result is in agreement with (Haleagrahara et al., 2011; Turdi et al., 2012). A reasonable explanation is that
NAC potentiates antioxidant ability of cardiomyocytes and restores glutathione status in cardiac tissue (Sochman, 2002; Rosic et al., 2015).

Finally, NAC improves LV function as detected by marked improvement in EF by 55.4% which is accompanied by increased LVESP, CO in association with decreased LVEDP and HR. These observations are consistent with that reported by (Ceylan-Isik et al., 2010; Hsu et al., 2006). In contrast to (Peake et al., 1996), found that adjunctive therapy with NAC in newly diagnosed septic shock patients is associated with a depression in cardiovascular performance. Taken together, an increase in cardiomyocytes contractility; and an attenuation of cardiac depressing factors such as TNF-α may be contributing factors participate in improvement of LV function by NAC.
4.2.2 Effects of Pre-Treatment with TAK 242 on Sepsis Induced Myocardial Injury

In this study, TAK 242 is used for the first time in attenuation of myocardial injury following sepsis. So TAK 242 is found to produce a significant decrease in both plasma and cardiac tissue levels of pro-inflammatory cytokines and chemokine (TNF-α, IL-1β, IL-6 and MCP-1). Similar findings were evidenced by (Ii et al., 2006; Sha et al., 2007; Kawamoto et al., 2008; Takashima et al., 2009; Zhao et al., 2015; Wang et al., 2016). Our data suggesting that TAK 242-mediated inhibition occurred largely through the TLR-4/NF-κB signal pathway (Hussey et al., 2013; Wang et al., 2016).

Regarding MMP-2 expression, this study shows for the first time that TAK 242 attenuates MMP-2 expression possibly through it is anti-inflammatory effect which mediated the inhibition of pro-inflammatory cytokines production. Consistent with (Ehrentraut et al., 2012) who found that administration of eritoran (TLR-4 inhibitor) improves cardiac function and limits the progression of myocardial hypertrophy likely by decreasing the pro-inflammatory cytokines, matrix metalloproteinase activity and increasing the anti-inflammatory cytokines.

Moreover, treatment with TAK 242 attenuates cardiac injury as evidenced by decreasing cTn-I. This finding is consistent with (Kaczorowski et al., 2007) who suggest that TLR-4 deficiency attenuates cardiac injury by decreasing the level of cTn-I (cardiac injury marker). Additionally, (Yousif and Al-amran, 2011) shows that in cardiomyopathy induced by trastuzumab, TLR-4 deficiency improves left ventricular function by causing marked increase in ejection fraction and cardiac output with significant decrease in cTn-I levels. Therefore, inhibiting or antagonizing TLR-4 could attenuate cardiac injury by decreasing cTn-I as shown in our study by using TAK 242.
In this study, it is found that administration of TAK 242 decreases the severity of cardiac histological changes (as compared with CLP). These observations are in agreement with (Zhang et al., 2015) who found that aldosterone treatment in rat causes cardiac injury and hypertrophy which is reversed by TAK 242 treatment. Also they found that TAK 242 significantly inhibits Aldosterone-induced perivascular fibrosis in the left ventricle. These results suggest that TAK-242 treatment suppresses cardiac inflammation and fibrosis which induced by Aldosterone-salt administration.

Consequently, TAK 242 administration results in the improvement of LV function by 41.1% increase in EF. This finding is in consistent with (Albertyn, 2012) who found that treatment with TAK 242 significantly restores the cardiac function in ischemia/reperfusion model.
Chapter Five

Conclusions & Recommendations
5.1 Conclusions

According to the results obtained from this study, we can conclude the following points:

1. This study is further supporting the notion that pro-inflammatory cytokines have a role in the pathophysiology of myocardial injury during sepsis.

2. This study supports the hypothesis that MMP-2 is up-regulated during sepsis and leads to LV injury making it a biomarker and a novel target for treatment of sepsis patients with myocardial injury.

3. Both NAC and TAK 242 may have a promising cardioprotective potential through down regulation of MMP-2 and inflammatory pathway.

4. The cardioprotective effect of NAC is better than TAK 242.
5.2 Recommendations

We recommend the following:

1- Study the signaling pathway by which NAC and TAK 242 suppress the MMP-2 expression.
2- Examine the combination effect of NAC and TAK 242 against sepsis induced myocardial injury.
3- Examine the post-treatment effect of both NAC and TAK 242.
4- Examine the effect of different doses level of each NAC and TAK 242 against sepsis induced myocardial injury.

5.3 Limitation of the study

The sepsis is included in many medical problems, such as systemic inflammatory response syndrome, and it is confirmed by many multiple factors that mediate cardiac tissue injury seen in the medical aspects. Additionally, analysis of the effect of one type of endotoxin can result in the understanding of the pathway by which this mechanism exerts its effect but CLP model could not be extrapolated to the complicated situation of sepsis and systemic inflammatory response syndrome. Furthermore, LV function was investigated by anesthesia and its effect may give some variability to the LV functional parameters.
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