

N-acetylcysteine attenuated sepsis-induced cardiac depression: down-regulation of MMP-2 pathway in miceNasser Ghaly Yousif^{1*}, Bassim I Mohammad², Musaed H. Al-Dahhan¹, Najah Hadi³, Safa Al-khalidy¹**Abstract**

During sepsis, the inflammatory responses mediate myocardium injury, including LV dysfunction and cardiac pathophysiological changes. To understanding the pathway of sepsis in cardiac depression, we tested the hypothesis that N-acetylcysteine attenuated sepsis-induced cardiac depression through down-regulation of MMP-2 pathway. Adult (4 to 6 months) male Albino-Webster mice, and their weights ranged from 25 to 30 gm, were pre-treated with N-acetylcysteine ip following cecal ligation and puncture (CLP). Left ventricle (LV) function was assessed using a micro-catheter system. MCP-1 and cytokines mediator's in plasma and myocardium were analyzed by enzyme-linked immunosorbent assay (ELISA). Further, the cardiac MMP2 measured by qRT-PCR and the pathological changes and cells injuries in the myocardium were examined using hematoxylin and eosin staining. CLP mice displayed worse LV function. The exaggerated cardiac depression in CLP mice was associated with higher levels of MCP-1 and cytokines in plasma and myocardium together with greater cardiac levels of cTn-I and MMP2. Neutralization of sepsis by NAC resulted in improved LV function and greater reductions in inflammatory mediators, MMP2 and myocardium injury. Taken together, NAC improved LV function following sepsis through down-regulation of MMP2 and serve as a potential therapeutic in cardiac endotoxemia.

Keywords: N-acetylcysteine; Myocardium injury; Sepsis*Corresponding Author: Nasser G. Yousif; email: yousif_ghaly@yahoo.com

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Sepsis induced myocardial dysfunction is the most common finding leading to increase the morbidity and the mortality. Its defines as a systemic

dysfunction (systolic and diastolic) of the left and right sides of the heart [1] which occurs due to the functional and structural injury in the myocardium

with or without lowered cardiac output [2] and it is characterizing by dilatation of the left ventricle and reduction in ejection fraction [3]. The occurrence of septic shock and multiple organs dysfunction syndrome in the septic patients is greatly due to myocardial dysfunction which leads to a decrease in the cardiac output, so this leads to vital organs hypo-perfusion, tissues' oxygen and nutrition supply reduction, immunity suppression; finally, organs dysfunction occurs [4].

Myocardium injury is a characteristic feature of endotoxemia and septic shock, which happening in about 40%-50% of sepsis [5]. In the intensive care unit, about 60% of severe sepsis patients exhibit cardiac dysfunction and the mortality for those patients range from 70-90%. In contrast, the mortality in patients who is not showing any signs of myocardial dysfunction due to sepsis is 20% [6]. There is great increment in the mortality of septic patients with left and right ventricles dysfunction [7]. The mechanism of sepsis induced myocardial dysfunction remains enigmatic.

Various cardio-depressants substances which have many mechanistic pathway (changes of homeostasis related to calcium, impaired of mitochondrial function, and disorder of myocytes apoptosis) that may be contributed in sepsis induced cardiac injury. Recently, found that the incidence of myocardial dysfunction appears to related with presence of TLR-4 on the cell membrane [8]. The TLR-4 mostly finds on macrophages, and a lesser extent on neutrophils, that triggered by sepsis induced myocardial injury [9].

When mice received Lipopolysaccharides (LPS), a rapid activation of nuclear factor (NF- κ B), with consequent increase of inflammatory cytokines; tumor necrosis factor (TNF- α) and interleukin-1 beta (IL-1 β) mRNA expression in cardiac cells are greatly ameliorating in TLR4-mutant mice [10]. These studies confirm that TLR-4 signaling is partly responsible for the stimulation of proinflammatory mediators in cardiomyocytes during sepsis. Cardiac extracellular matrix modulation occurs by many members of the large matrix metalloproteinase (MMP) family. So MMP has an important mechanistic focus in to the evolution of LV failure in both ischemic and non-ischemic disease [11].

Activation of MMP-2 is very important for regulation the proteolytic function for both sides of the cell membrane. Regulation of MMP-2 occurs at different parts including transcriptional, post transcriptional, and by involved with the endogenous tissue inhibitors (TIMPs) [11]. In cardiac cells, expression of MMP-2 can be actively up-regulated in response to reduced oxygen saturation, IL-1 β , or angiotensin II. Additionally, in the myocardial endothelial cells, MMP-2 expression and mRNA levels can be activated by inflammatory mediators. Expression of MMP2 occurs in normal myocardium cells, fibroblasts and blood vessels smooth muscle cells. In normal cardiomyocytes, MMP-2 locates in the subcellular compartments, including the mitochondria [12] thin and thick myofilaments of the cardiac sarcomere [13] cytoskeleton [14].

To understanding the pathway of sepsis related to sepsis induced-cardiac depression, we tested the hypothesis that N-acetylcysteine attenuated sepsis-induced cardiac depression through down regulation of MMP-2 pathway.

Materials and method

Experimental animals

Adult (4 - 6 months) male Albino-Webster mice and their weights ranged from 25 to 30 gm obtained from the College of Science, Babylon University. Murine were adapted for two weeks in light/dark cycle (12:12-h) with availability of free access to water and regular chow diet in animal house of Medical College, Kufa University and this investigation conforms to according to the Guide for the Care and Use of Laboratory Animals.

Sepsis procedure in mice

Cecal ligation and puncture (CLP) was established to induce endotoxemia in murine as described previously [15]. In brief, mice were anesthetized by i.p. injection mixture of ketamine and xylazine (Dreieich, Leverkusen, Germany). A 2-cm abdominal midline incision was performed to exposed cecum, ligated two-thirds of the cecum. We used 21-gauge needle to punctured the ligated part of the cecum, and repositioning the bowel with closed the abdomen by layers, using a 5.0 surgical suture (Norderstedt, Germany). Mice are monitoring every 3-hour/24-hour for various signs of sickness. Sham mice received anesthesia and laparotomy without CLP served as the surgical control group. The animals were assigned to the following

experimental groups: sham group, vehicle group, CLP group, CLP + Pretreated with NAC or TAC group ($n=8$ in each group). All treatments were performed in the morning and followed for survival for 24-hour.

Collection of samples

The samples of blood were drawn directly from the heart using needle, with heparin as anticoagulant and stored at 4°C. The blood centrifuged at $4700 \times g$ for 10 min at 4°C, and collected plasma stored at -20°C until used for further analyses. For heart collection, a thoracic operation was performed; the heart of the mice was excised. Myocardium tissues were cut into two parts: upper half of heart was snap-frozen until use, while the remaining parts were used for histological analysis.

Cardiac function measurements

We assessed cardiac function as described [16, 17]. Briefly, mice were anesthetized intraperitoneally with ketamin in dose of (50 mg/kg) post endotoxemia. Mouse was operated on supine position with heating blanket and maintained body temperature at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$.

A micro-tipped transducer catheter (1.4F, Millar Instrument Inc.) was placed into the right carotid artery to reached left ventricle (LV). While, the other part of the catheter was connected to machine for electrostatic chart recorder (Model ES 2000, Cleveland, USA). The pressure-volume loops recorded to measure changes in ventricular pressure and ejection fraction (EF%) through the MPVS-400

system and P-van software (Conductance Technologies, and Millar, Houston, TX) was applied to measure all data. Heart rates, left ventricle end-diastolic pressure (LVEDP), left ventricle systolic pressure (LVSP).

ELISA

The upper part of heart treated in PBS containing 0.5% Triton X100 with a protease inhibitor cocktail, tissue was homogenized and the supernatant used to quantify the chemokine and cytokines (MCP-1, TNF- α , IL-1 β , and IL-6) in both plasma and myocardial tissue, in addition to the plasma cardiac Troponin-I (cTn-I) according the instruction of commercial ELISA kits (R&D Systems).

The spectrophotometry of microplate reader (Bio-Rad Laboratories, USA) was used to determine the absorbance of standards and samples at 450 nm. All data were plotted and calculated against the linear portion of a reference standard curve [18].

Quantitative real-time PCR

The extracted total RNA from centrifuged supernatant homogenate myocardial tissue mixed by using Trizol-Reagent (Invitrogen, Carlsbad, CA), the equal amounts of RNA (1 μ g) were reverse transcribed by RNA PCR kit (Applied Biosystems, CA, USA) as described previously [19]. 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'-CAAGTTCCTCCCGGCGATGTC-3'

Antisense 5'-CTGGTCAAGGTCACCTGTC-3'
GAPDH Primer sequences
Sense 5'-AGAGAGAGGCCCTCAGTTGCT-3'
Antisense 5'-TTGTG GGGAGATGCTCAGTG-3'

Histological examination

The cardiac tissue samples were fixed in 4% paraformaldehyde for 24 h, as described previously [20]. Briefly, 5 μ m sections in thickness of cardiac tissue were paraffin embedded stained with the hematoxylin and eosin (H&E) through the standard procedure. The scoring of myocardial injury was examined according to the protocol of Zingarelli [21], its semi-quantify of the difference in cardiac damage in the histological sections from all mice groups ($n = 3$ sections per heart). The following criteria were used to scoring the myocardial injury: score 0, no damage; score 1 (mild), interstitial edema and localized necrosis; score 2 (moderate), diffuse myocardial cell swelling; score 3 (severe), the presence of contraction bands and neutrophil accumulation; and score 4 (highly severe), the presence of contraction bands, leukocyte infiltrate, and hemorrhage.

Statistical analysis

The data statistically analyzed by using StatView software (Abacus Concepts, USA) and to investigate differences between mice, and data differences ANOVA applied with Fisher post-hoc test that confirmed by using Mann-Whitney U-test. The survival curves were plotted by the Kaplan-Meier, and log-rank tests to evaluate the

differences. Statistically the present data significance was defined as $P \leq 0.05$.

Results

NAC improved LV function after CLP to investigate the treatment effects of NAC on LV function following sepsis by using CLP protocol. The ejection fraction, cardiac output and LVESP dropped rapidly in CLP and vehicle mice, in addition to elevated the LVEDP measurement, with significantly higher values of rising temperature by that time than in sham

group ($P < 0.05$) (Tab. 1). Furthermore, NAC pre-treated groups improved LV functions through increased the ejection fraction, cardiac output and LVESP beside reduced heart rate and LVEDP ($P < 0.05$). Furthermore, cardiac troponin I (cTn-I), as the one marker of cardiac injury, it is significantly up-regulation following CLP and vehicle-treated mice compared with sham ($P < 0.05$). Pretreatment with NAC significantly attenuated the adverse effects of sepsis on heart and reduced myocardial injury, cTn-I ($P < 0.05$; Fig. 1).

Table 1.

Hemodynamic status of mice treated with NAC for 24hrs CLP

Mice were subjected to CLP for 24h. Both I/R and I/R+vehicle mice displayed significantly reduced LV function. Treatment with NAC improved LV function. Data are expressed as mean \pm standard error, $n = 8$ in each group; * $P < 0.05$ versus sham; ** $P < 0.05$ versus I/R with or without NAC.

Groups	Heart rate (bpm)	LVEDP (mmHg)	Ejection fraction (%)	LVESP (mmHg)	Cardiac Output (ml/min)
Sham	422 \pm 14	3.3 \pm 1.2	63.1 \pm 2	121.1 \pm 1.2	5.3 \pm 1.3
CLP	478 \pm 21*	7.6 \pm 1.5*	32.5 \pm 1.5*	51.5 \pm 1.2*	3.0 \pm 1.4*
Vehicle	477 \pm 12*	7.6 \pm 1.4*	32.5 \pm 1*	51.2 \pm 1.4*	3.0 \pm 1.4*
NAC	462 \pm 12**	3.8 \pm 1.4**	55.4 \pm 1**	99.3 \pm 1.2**	4.9 \pm 1.4**

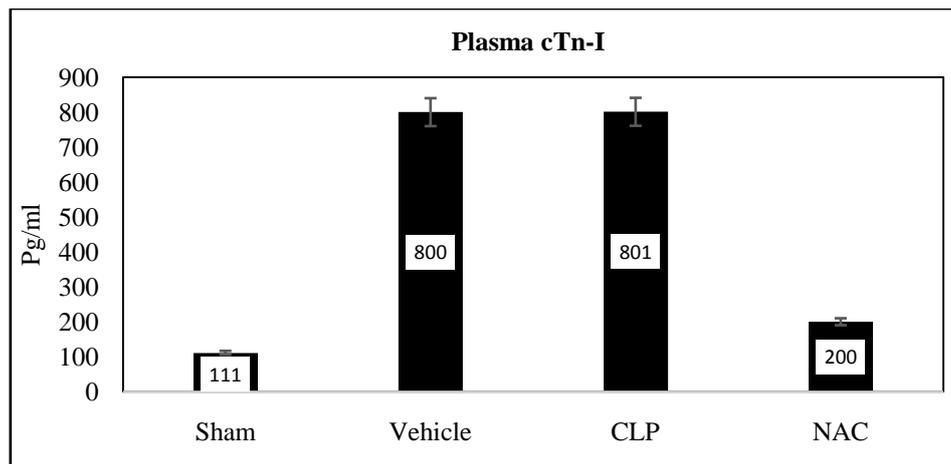


Figure 1.

NAC reduced cardiac injury.

Treated mice with NAC decreased level of the cardiac injury maker (cTn-I) after CLP. The mean of plasma cTn-I (pg/ml) of five experimental groups. Data are expressed as mean \pm standard error; * P <0.05 versus corresponding sham; ** P <0.05 versus CLP mice.

Effective role of pro-Inflammatory cytokines after CLP

We next investigated the importance effects of ANC on the local and systemic pro-inflammatory responses during CLP. At the end of the experiment (24 hrs after CLP), the levels of pro-inflammatory mediators including (TNF- α , IL-1 β , and IL-6) in myocardial tissue and plasma are measured by ELISA according to manufacture protocol. The resulted data showed that all pro-inflammatory cytokines are increased in both CLP and vehicle treatment mice group compared with sham group (P < 0.05) in both plasma and myocardial tissue. Furthermore, NAC significantly reduced the levels of all investigated cytokines compared with CLP and vehicle treated group, and this reflected improved the LV function (Fig. 2A, B).

NAC suppresses the expression level of MCP-1

Sepsis leads to upstream release of MCP-1 expression in plasma and myocardial tissue. Moreover, previous results demonstrated that MCP-1 have important role in the mechanistic pathology of myocardial injury by different pathways (Kumar et al., 2000). We assayed the expression of MCP-1 in plasma and myocardial tissue by ELISA. (Fig. 3A, B) showed that the levels of and MCP-1 are markedly increased by CLP, whereas NAC treatment attenuates both plasma and cardiac MCP-1 levels (P < 0.05).

MMP2-upregulated sepsis induced-myocardial suppression

To confirmed the crucial role of MMP2 in myocardium following sepsis and the possibility of down-regulation by pretreatment with NAC. The resulted data of qRT-PCR analysis showed that up-regulation of MMP2 in both CLP

and vehicle treated mice group. Furthermore, treatment with NAC down-regulated the MMP2 in myocardial cells was significantly ($P <$

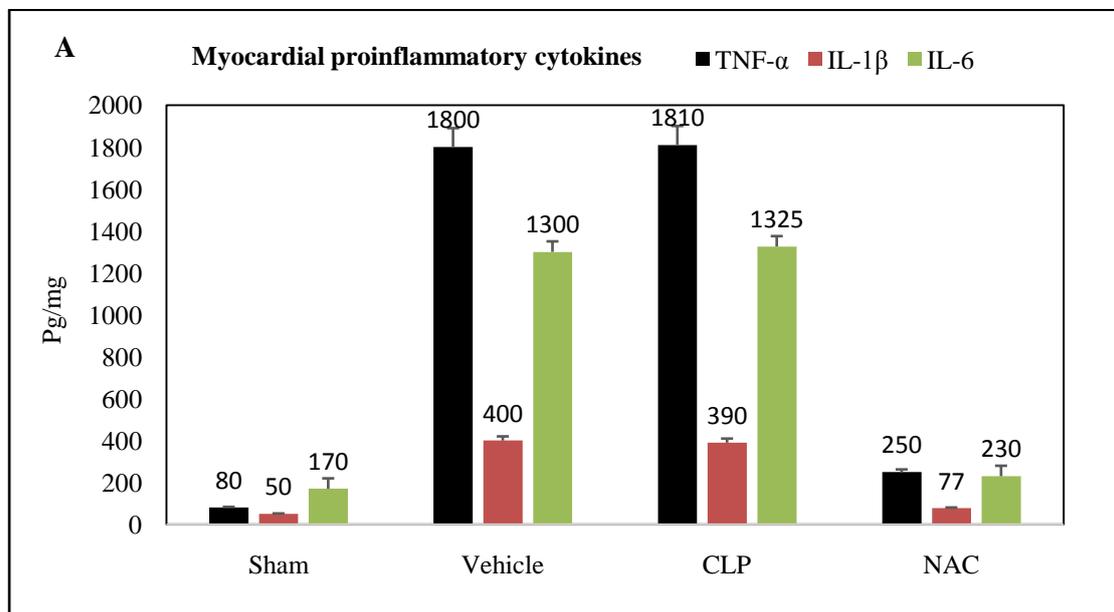
0.05). These data indicate involvement MMP2 in the mechanistic pathway of NAC (Fig. 4).

NAC treatment improved histopathological changes after sepsis and increased survival rate

Histological, myocardial tissue from CLP or vehicle mice after 24hr of sepsis period (Fig. 5A-C) revealed obvious myocardial injury with the development of contraction bands and polymorphonuclear leukocytes (PMN) accumulation besides interstitial edema and localized extravasations of red blood cells. While the histopathological changes in NAC treated group showed mild cellular alterations (Fig. 5D). According to Zingarelli scoring system; score 0 for sham mice group; 3-4

scoring to CLP alone and vehicle group; and score 2 (moderate) for NAC treated group (Fig. 5E).

The survival rate of mice in the CLP and vehicle groups was 90% during first 4hr and decreased to 60% through next 16hr. Pre-treatment with NAC results in improvement of the survival rate to 90 % during first 16 hours ($P=0.014$). The survival rates of mice were measured by the Kaplan-Meier method and compared by using the log-rank test (Fig. 6).



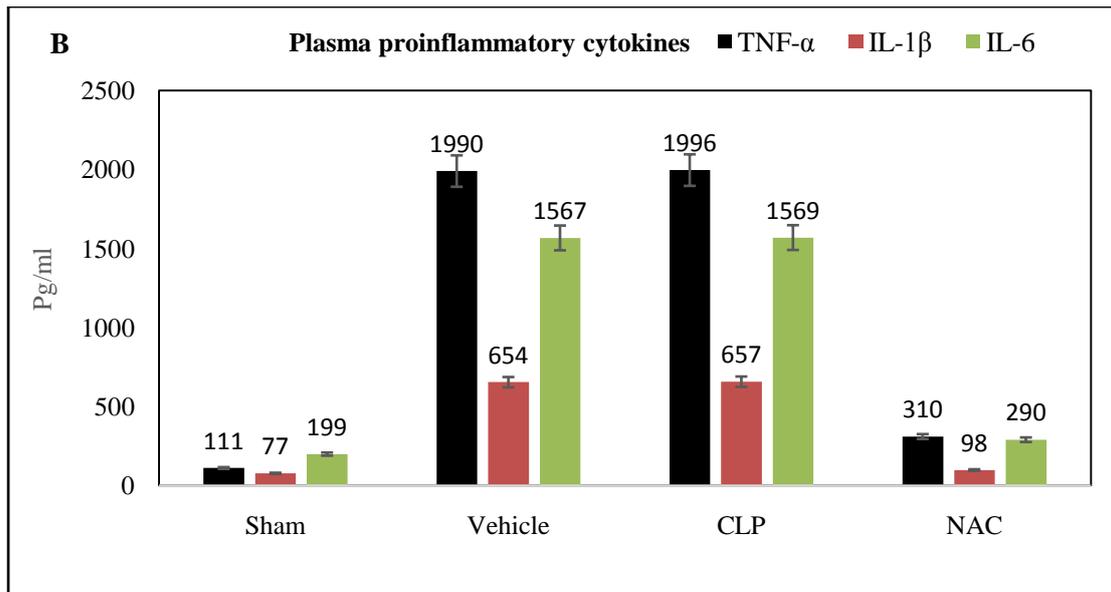


Figure 2.

Effective role of pro-Inflammatory cytokines after CLP

NAC suppressed the levels of cytokines (TNF- α , IL-1 β , and IL-6) after sepsis in both myocardium tissue (A), and plasma (B). The mean of myocardium pro-inflammatory cytokines (pg/mg) in the five experimental groups 24hr after CLP. Data are expressed as mean \pm standard error; * P <0.05 versus corresponding sham; ** P <0.05 versus CLP mice.

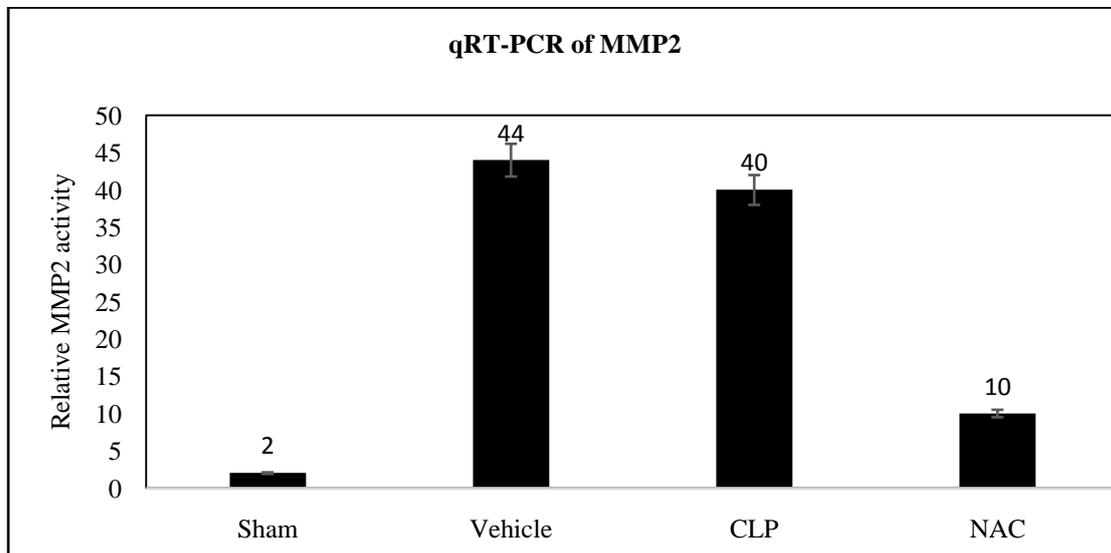


Figure 3.

NAC suppresses the expression level of MCP-1

Next investigate the effective role of NAC in reduction the levels of MCP-1 in both myocardium (A), and plasma (B). The mean of plasma MCP-1 level (ng/ml) in the five experimental groups 24 hrs after CLP. Data are expressed as mean \pm standard error; * P <0.05 versus corresponding sham; ** P <0.05 versus CLP mice.

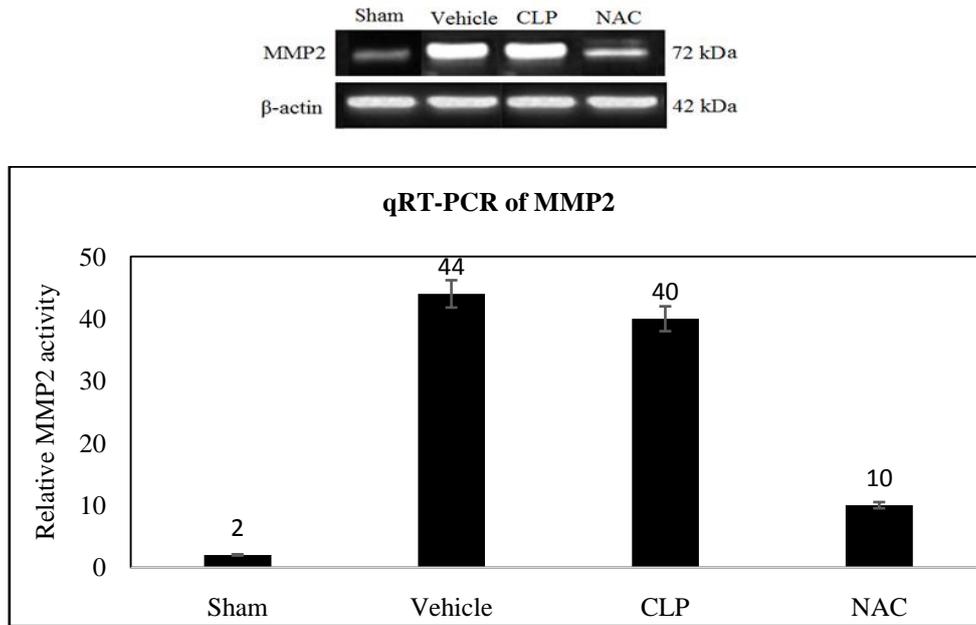
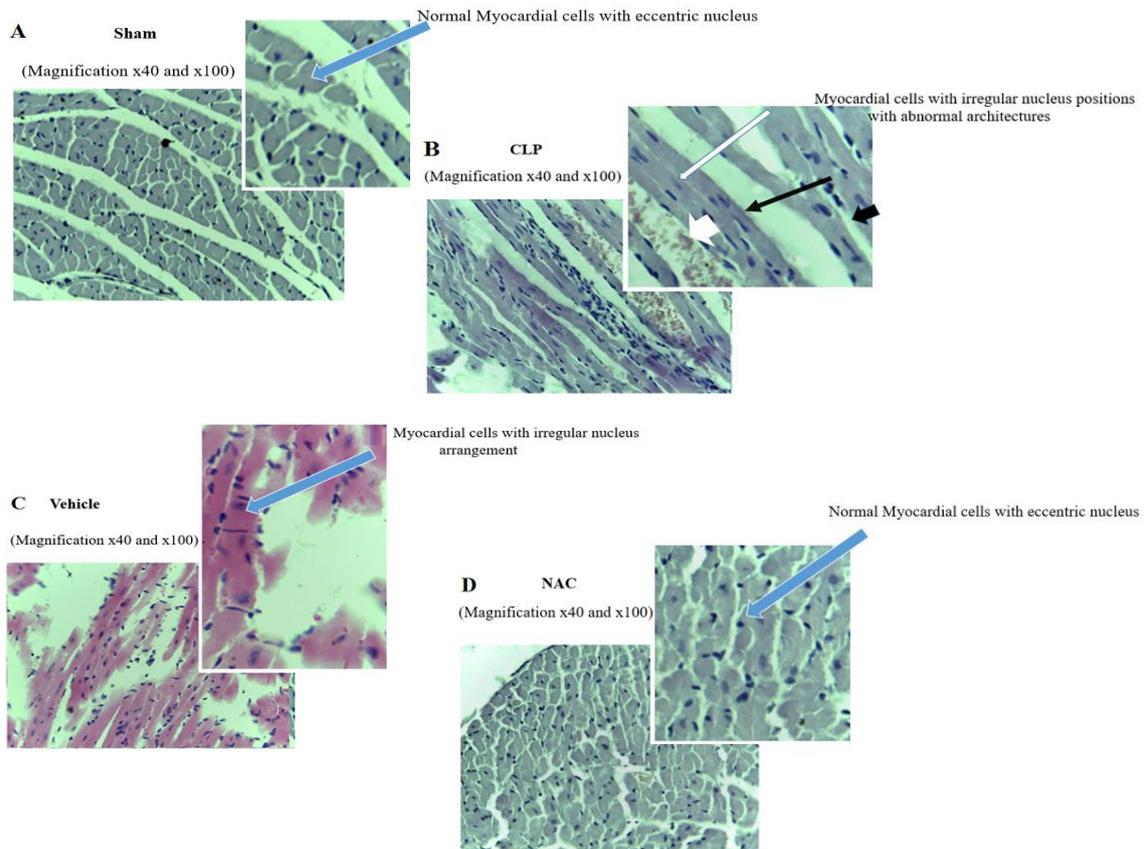


Figure 4.

MMP2-upregulated sepsis induced-myocardial suppression

Data of qRT-PCR showed that NAC down-regulation the MMP2 expression in cardiac tissue. The mean of relative MMP2 activity in the five experimental groups 24h after CLP. Data are expressed as mean \pm standard error; * $P < 0.05$ versus corresponding sham; ** $P < 0.05$ versus CLP mice.



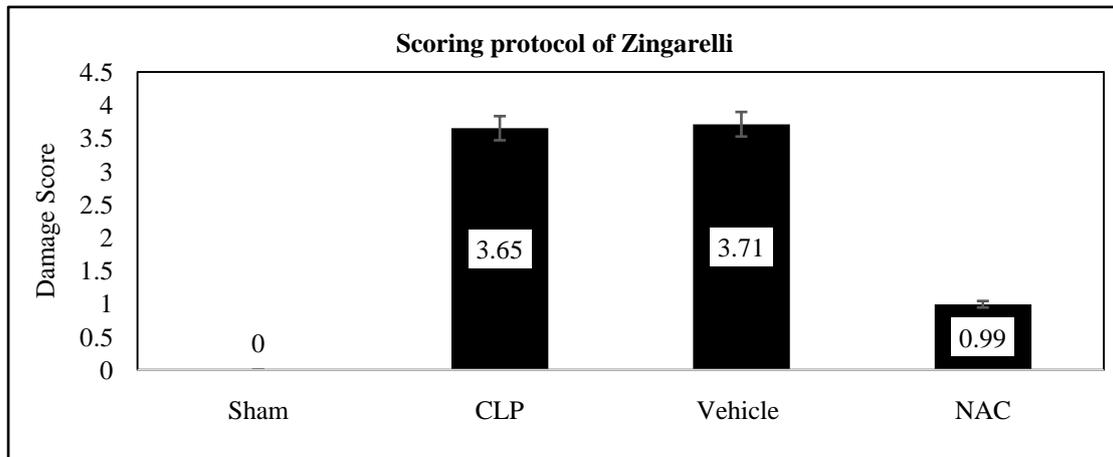


Figure 5.

NAC treatment improved histopathological changes after sepsis and increased survival rate Heart tissue from CLP demonstrating extensive contraction band change (black arrows) and extensive extravasation of red blood cells (white arrowhead) with migration of poly-morphonuclear leukocytes (PMN) (white arrows) and interstitial edema (black arrowhead) (A, B, C, D, E, F).

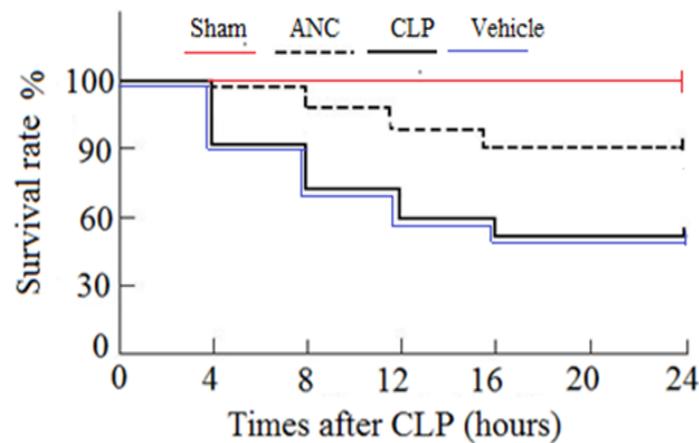


Figure 6.

The survival rates of mice groups were estimated by the Kaplan-Meier method and compared by using the log-rank test.

Discussion

During sepsis, the inflammatory responses mediate myocardial injury, including LV dysfunction and cardiac pathophysiological changes [22, 23]. Previous studies reported that expression of inflammatory mediators (IL-1 β , TNF- α and IL-6) was higher following myocardial injury and sepsis [24, 25]. It was also found that in vivo sepsis mice model and LPS-mediated enforced MCP-1 expression in both plasma and myocardial tissue [26].

To understand the pathway of sepsis related to the myocardial dysfunction, the present study investigated the NAC to improve the LV function following sepsis and possible pathway. According to our knowledge there was no data published discussed the relationship between MMP-2 pathway and effective role of N-acetylcysteine on improved cardiac function following sepsis by CLP model in mice. Sepsis attenuated myocardial function through inflammatory mediators.

A number of published paper have investigated and confirmed that myocardial dysfunction during sepsis is related with inflammatory mediator's expression, including IL-6, TNF- α and IL-1 β [27]. Furthermore, inflammatory mediators have been up-regulation after acute injuries caused by sepsis, myocardial ischemia, and burns [28].

Additionally, intravenous used of either IL-1 β or TNF- α in animal experiments evoke a similar process to that caused by sepsis lead to comorbidity and mortality, and this adverse effects of pro-inflammatory cytokines can be ameliorated by antibodies that antagonize the effects of

these molecules [29]. Other studies demonstrated that TNF- α also plays an important role in the septic myocardial dysfunction and that TNF- α links TLR4 activation pathway (. In the present study, we demonstrated that sepsis increases the levels of inflammatory mediators (IL-1 β , TNF- α and IL-6) in both plasma and cardiac tissue of mice, that associated with worse LV function performance through the hemodynamic measurements (heart rate, ejection fraction) and these results are associated with increased the levels of circulating cardiac Tn-I in mice exposed to CLP. Our data suggest that significantly higher levels expression of myocardial-depressant pro-inflammatory cytokines in the heart directly attenuated cardiac contractility and induce myocardial injury together of these results contribute, in some part, to the mechanism of exaggerated cardiac depression in experimental sepsis mice model.

Interestingly, we observed that pre-treatment with NAC results in a greater reduction in cytokines with improvement in LV function, ejection fraction was improved to (55.4 \pm 1%) in NAC treated mice, while the differences became much smaller in other LV functional parameters, such as LVESP, and cardiac output following treatment with NAC. Sepsis up-regulated myocardial MCP-1 expression level monocyte chemoattractant protein-1 (MCP-1) is one member of the C-C chemokine family, up-regulates the infiltration/migration of monocytes and neutrophils.

Many studies demonstrated that antagonized of MCP-1 has been shown to decreased neutrophils accumulation

and reduced injured tissue in many animal models of sepsis-induced organs injury [30]. In present data, we investigated that the expression MCP-1 levels in plasma and cardiac tissue are significantly higher in the CLP than sham mice, which is associated extensive contraction band change, extensive extravasations of red blood cells with migration of poly-morpho-nuclear leukocytes (PMN) and interstitial edema.

The overall pathological scoring indicates that NAC was significantly reduced the damage score compared with the CLP mice ($P < 0.05$, 3.5 ± 0.11 vs. $1 \pm 0.16\%$). In normal mammalian cells whether from tissues or organs, the cell cycle must be correctly controlled. Cell cycle regulation in cardio-myocyte is usually disturbed by a series of genetic changes, such as gene amplification, gene over expression or silence, and gene mutation. These genes include cell regulated genes such as MMPs that play important role in pathological changes in the aspects of both atherosclerotic plaque and myocardial injury [31, 32]. Therefore, the role of MMPs as a prognostic indicator can be through the factor that provokes acute myocardial injury changes. It is believed that MMPs pathway may act as a pivotal role in controlling cell injury by integrating different stimuli. If this pathway is correctly regulated in myocardial cell injury, we could target its some constituents such as MMP-2 by using some targeting inhibitor to reduce its effects. At present some of

these inhibitor molecules in this pathway have been considered as targets for experimental therapy, and some of them are under developmental way [33, 34]. Our study demonstrated that the expression MMP-2 is up regulation or it is stably expressed in sepsis-inducible myocardial injury, and deregulation status of MMP-2 expression is attenuate with NAC. We believe that MMP-2 may be also a potential up regulation of inflammatory mediators, and it needs more and more attentions to study its structure and function in the near future.

In conclusion, this work found that MMP-2 protein, one kind of MMPs, have endogenous expression after sepsis and lead to attenuated LV function. Further, it was found that the expression of MMP-2 is closely related to the changes of proinflammatory mediators (IL-1 β , TNF- α , and IL-6), further reduced myocardial function, and all of these suggest that MMP-2 could be a biomarker and a novel target for therapy in patients with sepsis to improved LV function. These experimental results let us believe that endogenous over expression of MMP-2 mediates the expression of MCP-1, led to increased level cTn-I with sequential signal caused myocardial cell injury. The qRT-PCR really showed that there were low levels of MMP-2 in treated ANC compared with no treated or vehicle mice. Although, we don't know why MMP-2 expression was suppressed under the effects of NAC, it remains to be further studies.

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. The CLP procedure and LV function measurement were investigated by used intraperitoneal injection anesthesia (ketamine) and its effect may give some different changes to the LV functional parameters.

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Authors' contributions

NG Yousif, BS, NH and SZ enrolled in the study design. NGY, SZ were participating in all parts of the study. All authors approved final version of manuscript.

Competing interest

The authors declare that there is no conflict of interest.

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