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Anti-inflammatory Effects of Atorvastatin in Polymicrobial Sepsis

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Abstract
Sepsis, a potentially fatal illness caused by infection, frequently leads to the failure of multiple organs, including the heart. The purpose of this study was to examine the possible mechanism of action of atorvastatin in improving cardiac function after sepsis. 35 Swiss albino male mice, weighing 30-38 g and aged 8–12 weeks, were divided in to five groups at random seven mice in each group, The control group continued to consume their regular food until the sampling time , while the sham group underwent laparotomy and anesthesia, the sepsis group underwent cecal ligation and puncture procedure while the vehicle group received an equivalent volume of intraperitoneal dimethyl sulfoxide (DMSO) injections for five days followed by cecal ligation and puncture procedure, and the Atorvastatin group received intraperitoneal injections of 20 mg/kg of Atorvastatin for five days after cecal ligation and puncture procedure ,Twenty hours after the cecal ligation and puncture ,The mice were euthanized and samples of serum and cardiac tissue were collected. The levels of serum TNF-α, IL-6, and CTN-I were assessed. The data, which followed a normal distribution, was examined using t-tests and ANOVA tests with a significance level of $p<0.05$. The group of sepsis exhibited considerably elevated levels of TNF-α, IL-6, and CTN-I compared to the sham group. However, the pre-treated group with Atorvastatin demonstrated significantly reduced levels ($p<0.05$) of these markers compared to the sepsis group. The histological characteristics of mice treated with Atorvastatin showed slight variations in comparison to control and sham groups.
Our inquiry findings suggest that Atorvastatin exhibits anti-inflammatory properties in cases of polymicrobial sepsis; this is supported by a significant reduction in the levels of TNF-α, IL-6, and CTN-I in the bloodstream.
KEYWORD: *sepsis, Atorvastatin, CLP, TNF-α, IL-6 and CTN-**I***

Introduction

Sepsis is a serious condition that results from an uncontrolled body response to an infection, resulting in a severe malfunction of the organs1. In 2017, sepsis caused over 11 million fatalities, resulting in an age-standardized mortality rate of 148 per 100,000 people. This accounted for nearly 20% of all deaths worldwide 2. Septic cardiomyopathy is a reversible impairment of the heart muscle that arises as a result of sepsis-induced multi-organ failure 3. The interaction between pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) with toll-like receptors (TLRs) on antigen-presenting cells (APCs) and monocytes lead to the transmission of signals that trigger sepsis. This, in turn, causes the movement of the nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) into the nucleus of the cell , This results in the expression of "early activation genes" which include several pro-inflammatory interleukins such as IL-1, IL-12, IL-18, tumor necrosis factor alpha (TNF-α), and interferons (IFNs). These then trigger the activation of

additional cytokines (such as IFN-y, IL-6, and IL-8), complement and coagulation pathways, and, through negative feedback, the suppression of components of the adaptive immune system 4. The early stages of septic illness can be characterized by an elevation in both pro-inflammatory and anti-inflammatory cytokines. The impact on the immunological phenotype, whether it leads to hypo-responsiveness or hyper-responsiveness, varies greatly from person to person and poses significant challenges for diagnosis 5 6 7.

Atorvastatin is a powerful, competitive inhibitors hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase 8. statins have significant pleiotropic effects, including enhancing endothelial function, reducing vascular remodeling, inhibiting Rho and its downstream target, and exerting anti-bacterial properties 9 10

AIM

This study aims to assess the cardioprotective effect of atorvastatin during polymicrobial sepsis in male mice.

Material And Methods

Animal Preparation

Thirty-five mature albino male Swiss mice were obtained from the Iraqi Center for Cancer Research. The mice weighed between (30 -38 g) were kept at the animal house of college Pharmacy at the University of Kufa. The mice were confined in enclosures that followed a 12-hour cycle of alternating light and darkness. The temperature in the enclosures ranged from 22 to 24 °C, while the humidity level ranged from 60 to 65%. The subjects had unlimited access to water and food. Institutional Animal Care approved the study and Use Committee (IACUC) of Kufa University, Research was conducted in the Laboratory of Clinical and Laboratory Department, college of Pharmacy, University of Kufa, spanning from December 5, 2023, to February 25, 2024,

Study design

The animals have been categorized into five groups, each consisting of seven animals, as shown below:

- Control group: The mice were provided with their regular diets until the time of sample collection.
- Sham group: Mice underwent laparotomy and anesthesia. The sham group represents the negative surgical control group.
- The Sepsis group: The mice underwent the cecal ligation and puncture (CLP) technique. sepsis group serves as the control group for surgical procedures with positive outcomes.
- The Vehicle group: The mice in this group were administered intraperitoneal injections of DMSO at a consistent amount for five consecutive days, after which they underwent CLP.

Experimental Model of Sepsis

Recent research has utilized mice to produce polymicrobial sepsis through the CLP model 11. The mice were anesthetized intraperitoneally with a combination of 100 mg/kg of ketamine and 10 mg of xylazine 12. The abdomen of the mouse was shaved and disinfected. Then, a midline incision of 1.5 cm was made for abdominal laparotomy, which exposed the cecum. Subsequently, the cecum was securely tied immediately below the ileocecal valve, punctured twice with a G-22 needle, and softly compressed to extract a small amount of stool from the puncture before being returned to its original position. Subsequently, a 5/0 surgical suture was employed to seal the abdominal incision 13. By administering a subcutaneous injection of 1 ml of a 0.9% saline solution, the mice were revived, subsequently; the mice were monitored every 4 hours for a duration of 24 hours before being returned.

Atorvastatin preparation

Atorvastatin was obtained as pure powder from med chem express,CHINA. Atorvastatin was solubilized in dimethyl sulfoxide (DMSO). Subsequently, a dose of 20mg for each kg of atorvastatin was delivered intraperitoneally for a duration of five days 14 , following the CLP procedure.

SAMPLES COLLECTION

BLOOD SAMPLES

Blood samples were gathered by heart puncture before sacrificing mice. A gel tube was placed and kept for one hour at room temperature. The serum was separated by centrifuging blood for 20 minutes at 4000 rpm. The amounts of CTN-I were quantified using the enzyme-linked immunosorbent assay (ELISA) method

Tissue preparation for ELISA assessment of TNF-α and IL-6

The cardiac tissue were washed with a solution of 0.9% sodium chloride to eliminate any traces of blood before being preserved at a temperature of -80 degrees Celsius in a deep freezer. The heart sections were homogenized using a mortar and pestle and then processed with a high-intensity ultrasonic liquid processor in a phosphate buffered saline solution containing 1% TRION X-100 and 1% protease inhibitor cocktail 15. The tissue homogenate was centrifuged at a speed of 3000 revolutions per minute for 20 minutes at a temperature of 4 degrees Celsius. Subsequently, levels of TNF-α and IL-6 were measured in the supernatant of the tissue homogenate in accordance with the manufacturer's instructions for ELISA kits.11

Tissue Preparation for Histopathology

The cardiac tissue collected after sacrificing the mouse was washed with a cold solution of isotonic sodium chloride (0.9 percent) .The heart tissue was fixed in a 10% formaldehyde solution for a duration of 20 hours. Following the process of dehydration and cleaning, the heart tissue was placed in a paraffin block and then sliced into a 5 µm-thick segment using a microtome. The tissue samples underwent staining using the hematoxylin and eosin (H&E) technique 16

Histological Examination

Cardiac damage was evaluated for each segment of the heart using an optical microscope, and images of the sections were recorded. Utilizing Zingarelli's methodology, histological sections from all groups were evaluated and categorized to approximately ascertain the degree of difference in heart injury17. The parameters for this evaluation method were:

- Score zero: there is no harm or destruction: (normal tissue).
- Score 1: a localized necrosis and interstitial edema (mild).
- Score 2: a Swelling of cardiac cells and widespread necrosis (moderate).
- Score: 3 an Ischemia with an accumulation of neutrophils: (severe).
- Score 4: contractile bands, bleeding, ischemia, and leukocyte infiltration (very severe).

Statistical Analysis

Graph Pad Prism version 8.1 was used to do the statistical analysis. The data was displayed using the mean ± standard error mean (SEM). one-way analysis of variance (ANOVA)test was used in this investigation to assess The disparities between the experimental groups. Post hoc tests were then performed using Bon ferroni several comparisons method. Dunn's post hoc testing and non-parametric tests were used to compare the

histopathological alterations between the groups. In statistics, a test was deemed significant if the P value achieved was less than 0.05.

RESULTS

The Effect of Atorvastatin Treatment on TNF-α Level

Compared to the mice in the sham and normal groups, the sepsis group's serum TNF-α levels were considerably higher ($p<0.05$).

There was no discernible disparity in the levels of TNF-α, in the serum between the vehicle and sepsis groups, as well as between the sham and normal groups (Fig.1). The group administered with atorvastatin had a significant reduction in blood TNF-α levels in comparison to the sepsis and vehicle groups ($P<0.05$).

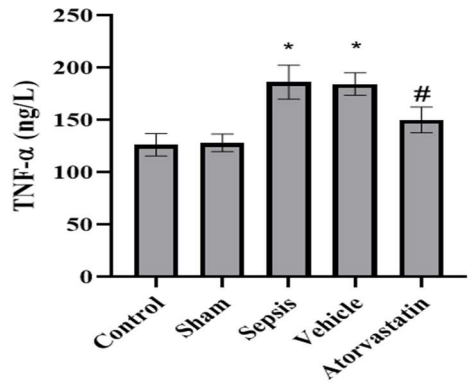

Figure 1: the Serum level of the TNF-α in experimental group.
*: Significantly, different ($p<0.05$) compared to control or Sham group.
#: Significantly different ($p<0.05$) compared to control or sepsis or vehicle group.

The Effect of atorvastatin Treatment on IL-6 Level

The mice in the sepsis group exhibited markedly elevated blood IL-6 levels compared to the mice in the sham and normal groups ($P< 0.05$). There was no statistically significant variation in serum IL-6 levels reported between the vehicle and sepsis groups, as well as between the sham and normal groups (Fig.2). The group administered with atorvastatin had a significant reduction in blood IL-6 levels in comparison to the sepsis and vehicle groups ($P<0.05$).

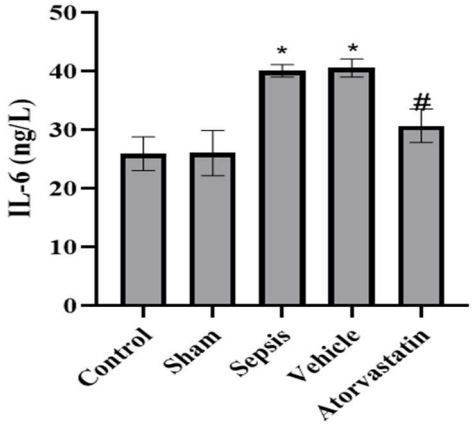

Figure 2: the Serum level of IL-6 in experimental groups. *: Significantly, different ($p<0.05$) compared to control or Sham group.
#: Significantly different ($p<0.05$) compared to control or sepsis or vehicle group.

The Effect of Atorvastatin Treatment on CTN-I Level

The mice in the sepsis group exhibited markedly elevated blood CTN-I levels compared to the mice in the sham and normal groups ($P< 0.05$). There was no statistically significant variation in serum CTN-I levels reported between the vehicle and sepsis groups, as well as between the sham and normal groups (Fig.3). the group treated with atorvastatin exhibited a notable decrease in blood CTN-I levels compared to the sepsis and vehicle groups ($P<0.05$).

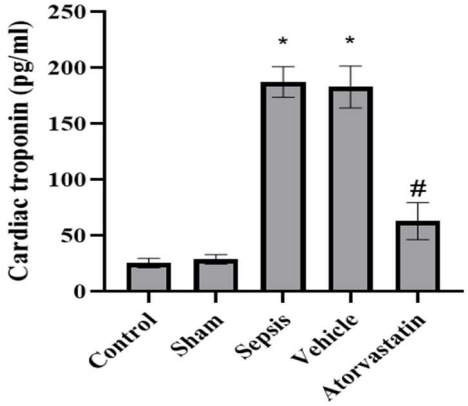

Figure 3: the Serum level of CTN-I in experimental groups.
*: Significantly, different ($p<0.05$) compared to the control or Sham group.
#: Significantly, different ($p<0.05$) compared to the sepsis or vehicle group.

HISTOPATHOLOGICAL CHANGES OF MYOCARDIAL TISSUE AFTER POLYMICROBIAL SEPSIS

Myocardium tissue of the normal and sham groups showed the tissue of the heart had normal architecture, with defined borders between the myocytes and no erythrocyte leakage or leukocyte infiltration (fig.4. A-B). Every mouse in both normal and sham group exhibits typical histopathology results (score 0) as shown in (Fig. 5).

The sepsis and vehicle groups both have sever myocardial damage (score 3) characterized by a severe combination of acute and chronic inflammatory cell infiltration, consisting of neutrophils and lymphocytes. Mild edema and significant congestion of blood vessels accompanied this infiltration. (Fig.4.C-D). the mean histological score was significantly higher in the vehicle and sepsis groups than in the normal and sham groups ($P<0.05$), (fig.5).

Atorvastatin –treated group had mild myocardial injury (score 1) (fig. 4E). The average histopathological score was lower in the atorvastatin-treated group compared to the sepsis and vehicle groups ($P<0.05$), (fig 5).

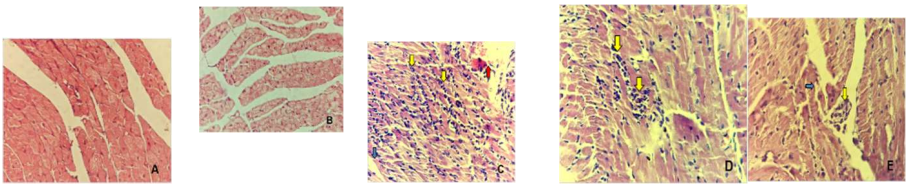

Fig4: Photograph of the cardiac section that stained with H&E (X400). A: normal group showed normal architecture (score 0); B: the sham group showed normal architecture (score 0); C: the sepsis group, showed inflammation (yellow arrows), edema (blue arrows) and congestive blood vessel (red arrow); D: the vehicle group showed inflammation (yellow arrow); E: the Atorvastatin group showed mild inflammation (yellow arrows) and mild edema (blue arrow)

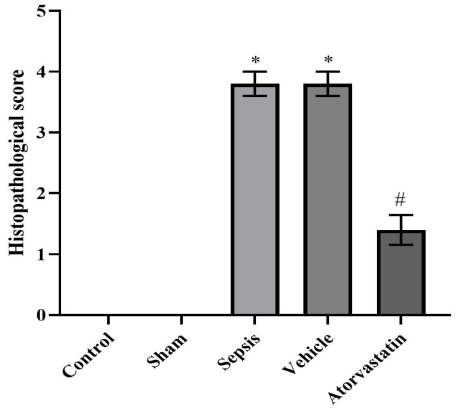

Figure 5: Histopathological scores in experimental groups.

* Significant versus sham or normal groups (P<0.05).
. # Significant versus sepsis or vehicle groups (P<0.05).

DISCUSSION

Sepsis-induced cardiac injury is referred to as septic cardiomyopathy, and it is linked to higher rates of illness and death. The heart is unable to maintain sufficient pre load due to hemodynamic abnormalities caused by sepsis 7. Sepsis is characterized by the dysregulated host response, where the disease is caused by a combination of pathogen-related factors and immune-cell-mediated inflammatory responses, which may have either deleterious effects at early or late stages of the illness 18. Cytokines, especially TNFα and IL-6, activate the coagulation cascade, resulting in expression of the tissue factor on the surface of cells 19 20.

Significantly higher serum levels of TNF-α were found in the vehicle and sepsis groups compared to the normal and sham groups in this study. In contrast, the Atorvastatin -treated group had markedly lower TNF-α serum levels than the vehicle and sepsis groups. 21And 22 were found that CLP caused an elevation in the production of TNF-α, which led to significant tissue damage.in contrasts 23 demonstrated that rats undergoing CLP operation and treated with Atorvastatin exhibited significantly lower levels of TNF alpha compared to the sham group .also 24 had provided the same results.

This study also discovered markedly elevated serum levels of IL-6 in the vehicle and sepsis groups in comparison to the control and sham groups In contrast, the Atorvastatin -treated group had markedly lower IL-6 serum levels than the vehicle and sepsis groups. 25 demonstrate that compared to sham-CLP operated animals,

septic rats had elevated amounts of IL-6 and TNF-α in the bloodstream. Subsequent to the administration of atorvastatin. 26 observed that levels of IL-6 and TNF-α in the serum were elevated in septic animals in comparison to rats that underwent a sham operation. Following the administration of atorvastatin, there was a notable reduction in circulating levels of IL-6 and TNF-α

Furthermore, serum levels of CTN-I were considerably elevated in both the vehicle and sepsis groups as compared to the normal and sham groups. In contrast , the Atorvastatin -treated group had markedly lower CTN-I serum levels than the vehicle and sepsis groups .27 showed that, at 18 hours following CLP induction the serum cTn-I levels were significantly higher in septic animals with cardiac dysfunction compared to a sham group while On the other hand , 28 demonstrated that simvastatin -treated rats have a lower CTN-I level than rats with sepsis induced by cecal ligation and puncture (CLP) .

For sepsis and vehicle groups, the majority of the histopathological damage scores were quite severe (score 3). On the other hand, when compared to sepsis and vehicle groups, atorvastatin treatment dramatically minimizes cardiac tissue injury. The group that received atorvastatin showed histopathological damage levels that were mild (score 1). 29 showed that an atorvastatin pretreatment significantly reduced neutrophil accumulation and oxidative stress. When taking atorvastatin, reduced glutathione levels seemed to return to a roughly controlled level. Additionally, the pretreatment reduced histopathological-level mucosal damage 30 found that administering atorvastatin intravenously during ischemia lowers senescence, apoptosis, and Cardioprotective / metabolic, associated indicators. Demonstrated that H and E-stained slices from the Atorvastatin group of male albino rat showed cardiomyocytes with characteristic striated branching architecture, acidophilic cytoplasm, central oval vesicular nuclei, and interstitial blood capillaries.

Conclusion

The study's results confirm the concept that Atorvastatin possesses notable anti-inflammatory and Cardioprotective characteristics, effectively reducing cardiac harm caused by sepsis.

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