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AMPK–NRF2–MEDIATED CARDIOPROTECTION BY LUTEOLIN IN A MURINE MODEL OF ENDOTOXIC MYOCARDIAL DAMAGE

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Abstract

Multi-organ failure brought on by the body's aberrant reaction to microbial invasion is the hallmark of polymicrobial sepsis, a potentially fatal condition. The study aimed to elucidate the putative cardioprotective action of luteolin against endotoxic heart injury induced by experimental sepsis in mice. Experimental animals were divided in to four groups, each contain 6 mice. Cecal Ligation and Puncture, Sham, DMSO & luteolin-treated groups 50mg/kg IP, 1hour before Cecal Ligation and Puncture, after that animals were sacrificed 24 hours after Cecal Ligation and Puncture. Markers evaluated include, IL-1β, TNFα, TNF αr1, HO-1 F2-ISOPROSTANEMPO, CASPASE-11 & troponin by ELISA technique, histopathological study & PCR for gene expression of AMPK-Nrf2 was done. IL-1β, TNFα, TNFαr1, HO-1, MPO, CASPASE-11, F2-isoprostane, tissue mRNA expression of AMPK-Nrf22 genes were significantly altered in the luteolin-treated group compared to the CLP group ($p<0.05$). We conclude that Luteolin contains anti-inflammatory and anti-oxidative properties; we conclude that it has cardioprotective benefits. Additionally, luteolin demonstrated a cardioprotective impact by influencing the AMPK-Nrf2 genes' tissue mRNA expression.

Keywords*:* **CLP, Sepsis, Luteolin, oxidative stress, apoptosis, inflammation and cardio protection**

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Introduction

Sepsis is a global health problem resulting from a dysregulated host response to infection [1]. It stands as a leading cause of mortality subsequent to infections. The pathogenesis of cardiomyopathy in the context of sepsis involves intricate alterations in molecular, metabolic, and even structural aspects within the myocardial cells [2]. One important consequence of sepsis is cardiac dysfunction, which raises death rates. Increased inhibition of glucose and fatty acid oxidation by inflammation, depletion of ATP & diminishing of the heart sympathetic response have all been connected to these phenomena [3]. Another mechanism by which sepsis impairs cardiac function is increasing the production of pro-inflammatory cytokines such TNF-α, IL-1β, and IL-6, act as pro-inflammatory mediators and cardio-depressant resulting in cardiac contractile dysfunction, cardiac atrophy at the end lead to heart failure [4,5]. AMPK acts as an intracellular energy sensor and plays an important role in

regulating inflammatory responses. Recent research suggests that AMPK activation might reduce oxidative stress and reduce inflammation [6,7]. Thus far, The NF-κB pathway has been the main focus of the mechanistic connections between AMPK and inflammation. Research indicates that AMPK chemical activators can reduce NF-κB-mediated transcription [8]. The possibility of interactions between the AMPK and Nrf2 and pathways was recognized due to the documented function of the Nrf2 pathway in reducing inflammation and oxidative stress [9], however, there is currently a lack of data concerning the possible interactions between the Nrf2 and AMPK pathways within the inflammatory system of mammals [10]. The signaling pathways included in the inflammatory response are arranged in to anti-inflammatory pathways and proinflammatory pathways and the former involved Nrf2 pathway and the latter includes NF-κB pathway [8]. Luteolin exhibits antioxidant, anti-tumor, anti-inflammatory, and anti-apoptotic properties [11]. Given its robust anti-tumor and anti-inflammatory characteristics, Luteolin effects on vascular vessels and the heart have been investigated [12].

This research aimed to elucidate the putative cardioprotective action of luteolin against endotoxic heart injury induced by experimental sepsis in mice.

Materials and methods

Study design

Adult Swiss white mice, 24 males (weighing 25–35 g, aged 4-8weeks) were purchased from the animal resource center, with alternative 12-hrlight/12-hr dark cycles, under temperature of 24°C ± 2°C, free access to diet and water. Animals were divided randomly into 4 groups (n=6) : Sham group: All mice underwent identical anesthesia and surgical procedures (laparotomy incision) for the same duration as the sepsis-inducing operation, but without actually inducing CLP , DMSO group (vehicle control)received DMSO 10 % (v/v) as standard vehicle for luteolin then underwent CLP procedure for 24hr,CLP group Cecal ligation and puncture (CLP) group: Mice underwent median laparotomy incision under anesthesia, followed by cecal ligation and puncture for 24 hr, and luteolin treated group : mice were pretreated with luteolin (50 mg/kg IP), one hour before CLP; animals were euthanized twenty-four hours following CLP [13] .

Experimental procedure

Based on earlier research, the cecal ligation and puncture paradigm was used for inducing sepsis [14]. In summary, during the first 24 hours of sepsis, we used 18-G needle with the twofold puncture technique to induce organ (cardiac) dysfunction. To make sure the puncture wounds were open, a tiny bit of stool was removed. The abdomen was then sutured. In order to cause organ (cardiac) malfunction within the first 24 hours of sepsis, all animals were given a subcutaneous resuscitative normal saline dose 20 mL/kg body weight [15].

Luteolin solution preparation

Luteolin was obtained from MACLIN company, China, it is diluted in 10% DMSO, then administered in dose of 50 mg/kg intraperitoneally hour before CLP [16].

Samples collection

After (24 hours) from procedure end, mice anesthetized by ketamine/ xylazine 100/20 mg/kg. A 1–1.5 ml sample of blood was taken right away from the heart. After that, the blood samples were centrifuged for ten minutes at 3000 RPM. Red blood cells were eliminated by centrifuging the serum once more for one minute at 3000 RPM [17-19]. The cardiac tissue was washed by saline in order to remove RBCs clots and divided into 3 portions, one for homogenization & ELISA, another for qRT- PCR & the last portion was preserved in formalin till a histological examination was carried out.

Measurement of TNFαR, IL-1β, TNF-α, HO-1, MPO, CASPASE-11 &F2-ISOPROSTANE of Tissue homogenate

The cardiac tissue was homogenized using a high-intensity ultrasonic liquid processor in a ratio 1:10 (w/v) PBS contain 1% Triton X-100 and a protease inhibitor cocktail then rinsed with ice-cold normal saline to remove RBCs or clots. The homogenates centrifuged for 20 minutes at 4°C at 3000 rpm. The supernatant was collected in order to evaluate the levels of IL-1β (Catalog Number: E0192Mo), F2-isoprostane (catalogue no: E2719Mo), TNF α (catalogue no: E0117Mo), TNFαR (Catalog Number: E2539Mo), HO-1 (catalogue no: E0266Mo), MPO (catalogue no: E0436Mo), and Caspase-11(catalogue no: E1512Mo), using the ELISA technique (BT lab china) [20].

Histopathology Tissue preparation

The Zingarelli protocol was followed for the histology and grading of the cardiac tissue [21].

qRT-PCR analysis of AMPK-Nrf2 heart tissue expression

According to the manufacturer's instructions, a quantitative real-time PCR is used for measuring the mRNA expression of AMPK-Nrf2. The heart tissue was rinsed with an ice-cold saline to remove any red blood cells or clots and then kept in RNA later solution (TriRNA Pure Kit), until homogenization with a high-intensity ultrasonic liquid processor in 1:10 (w/v) PBS that contained 1%Triton X-100 and a protease inhibitor cocktail. The homogenates were centrifuged at 3000 rpm for 20 min at 4°C The supernatant was collected for RT-PCR. Total RNA was extracted using specialized reagents and equipment for real-time quantitative (RT-q) PCR. The primer sequences used for q RT-PCR are:

AMPKf: 5-CTCTATGCTTTGCTTTGCTGTGTGG-'3

AMPKr: 5-GGTCCTGGTGGTTTCTGTTG-3'

Nrf2f:5-CAGCATGATGGACTTGGA-3'

Nrf2r: 5-TGAGAGACTGGTCACACT-3'

Statistical analysis

SPSS version 24.0 for windows was used. Results are presented as mean ± SEM. Comparisons among the experimental groups were done using one-way ANOVA and post hoc Bonferroni test. Histopathological scores (0–4) were tested by Kruskal-Wallis & Mann-Whitney U tests Schapiro tests were used after testing normality of data. $P<0.05$ was considered statistically.

Results

Inflammatory markers IL-1β, CASPASE-11, TNF-α, TNFαR, MPO, and oxidative biomarkers HO-1 & F2-ISOPROSTANE were found to be significantly elevated ($p<0.05$) in the DMSO and CLP groups while compared to the sham group (significant difference P value <0.05). Furthermore, when compared to the CLP & DMSO groups, the luteolin group displayed significantly lower levels of these markers ($p<0.05$). More intriguingly, the sepsis and DMSO groups had significantly greater serum troponin levels ($p<0.05$) than the sham group. Additionally, the luteolin group's serum troponin levels were significantly lower than those of the sepsis and DMSO groups (significant difference P value <0.05),

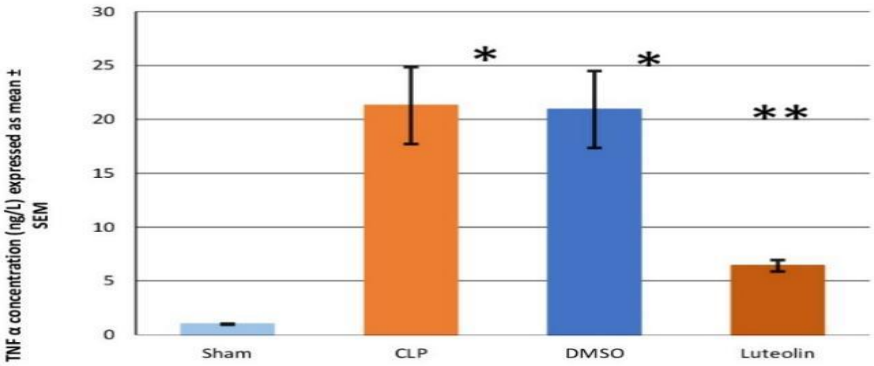


Figure 1: TNF-α (ng/L) mean tissue level of Data as expressed as mean ± SEM

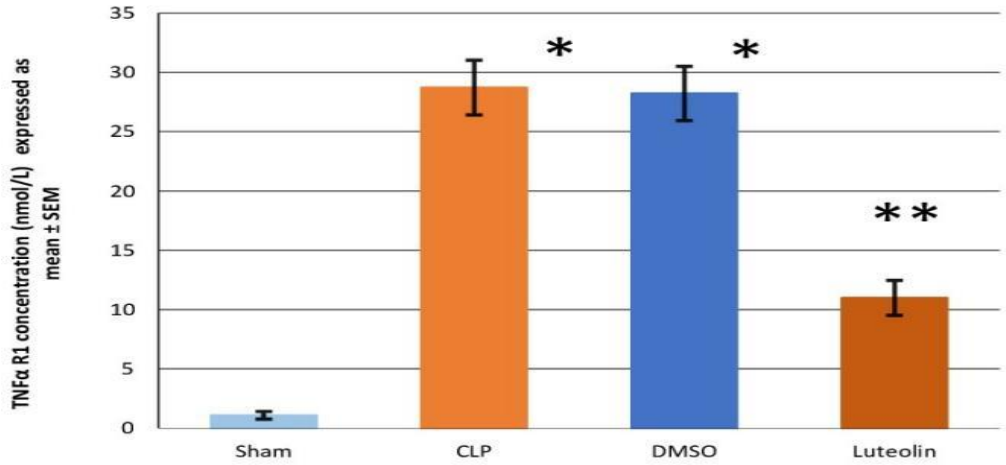


Figure 2: TNF-αR1 (nmol/L) mean tissue level of Data as expressed as mean ± SEM

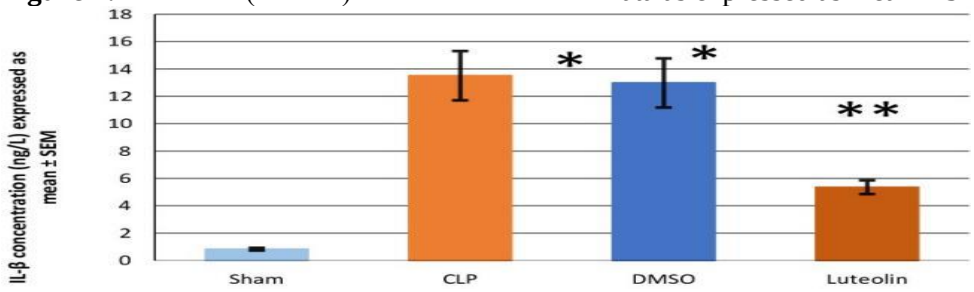


Figure 3: IL- 1β (ng/L) mean tissue level of Data are expressed as mean ± SEM

Figure 4: Caspase-11 (pg/ml) mean tissue level of Data are expressed as mean ± SEM

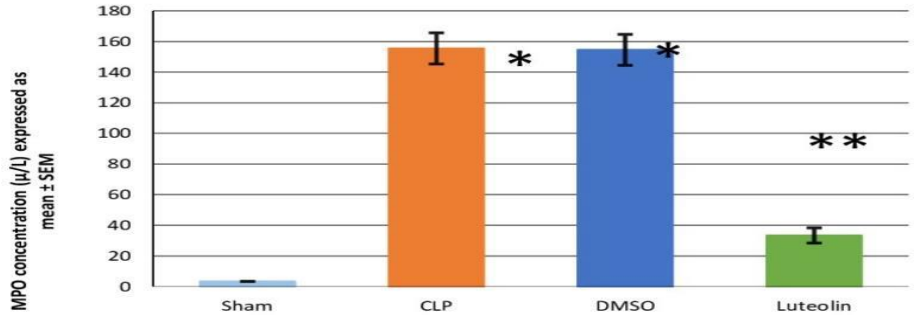


Figure 5: MPO (µ/L) mean tissue level of Data are expressed as mean ± SEM

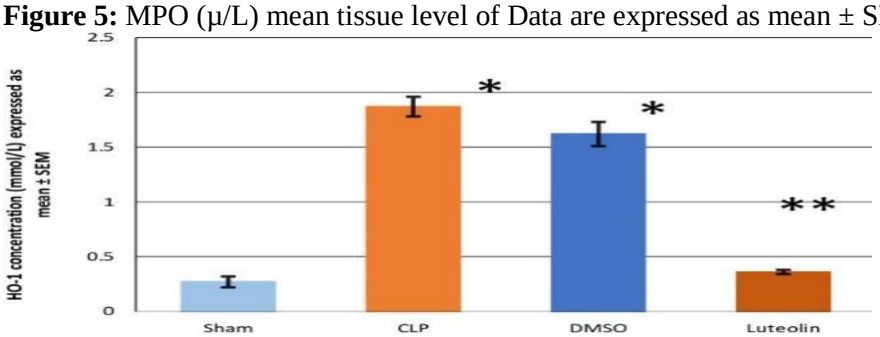


Figure 6: HO-1 (mmol/L) mean tissue level of Data are expressed as mean ± SEM

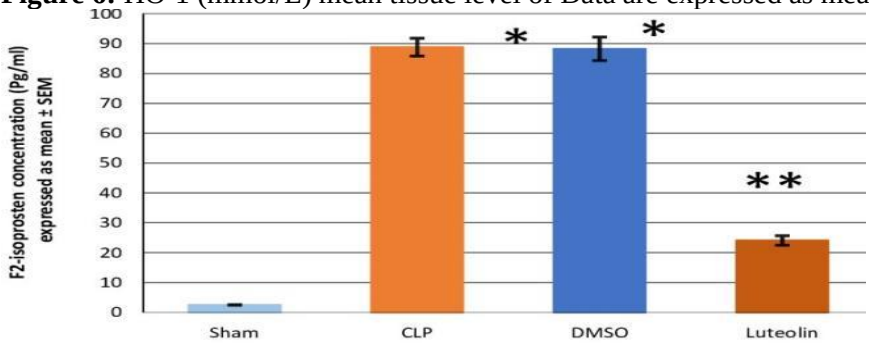


Figure 7: F2- isoprostane (pg/L) mean tissue level of Data are expressed as mean ± SEM

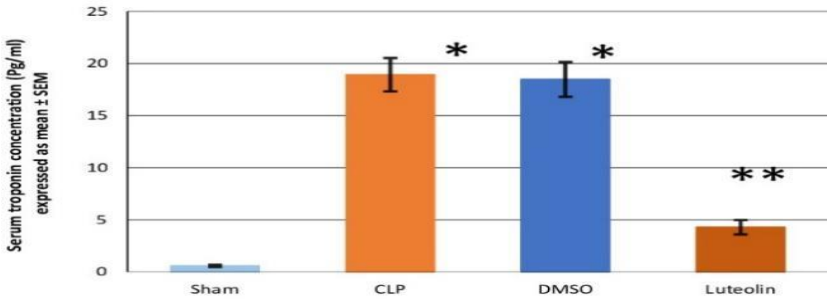


Figure 8: Troponinn (pg/ml) mean serum level of Data are expressed as mean ± SEM

Luteolin's impact on AMPK-Nrf2 mRNA expression

When compared the CLP and DMSO groups with sham group, quantitative real-time PCR showed a substantial decrease in the mRNA expression of the AMPK-Nrf2 gene ($p<0.05$). The luteolin group showed significantly increased levels of AMPK-Nrf2 ($p<0.05$) compared to the CLP and DMSO groups (significant difference P value <0.05).

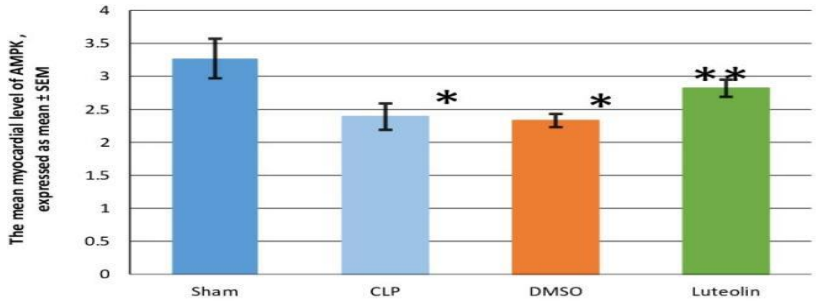


Figure 9: AMPK expression mean myocardial level of Data are expressed as mean ± SEM

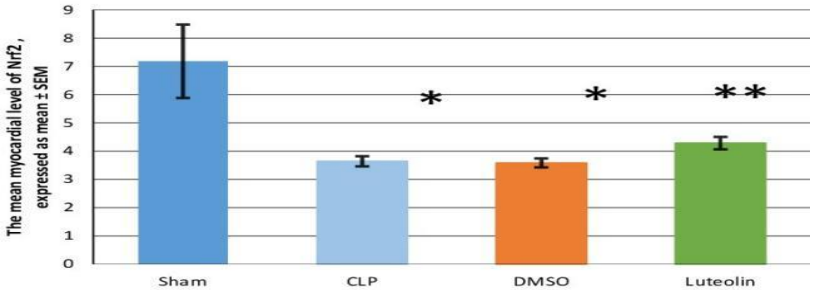


Figure 10: Nrf2 expression mean myocardial level of Data are expressed as mean ± SEM.

For all figures

*: Significant difference in (CLP& DMSO) as compared with the sham group

**: significant difference in luteolin group as compared with CLP group

Histopathotogical findings

According to the data, CLP significantly damaged tissue, as indicated by scores ranging from 0 to 4. The Sham group (A) displayed normal cardiomyocytes, whereas the CLP and DMSO groups (B) scored 4, indicating necrotic cardiomyocytes. By decreasing the necrosis in the cardiomyocyte figure (11), luteolin group (C) score 2 demonstrates a decrease in tissue damage.

Sham group: (A)

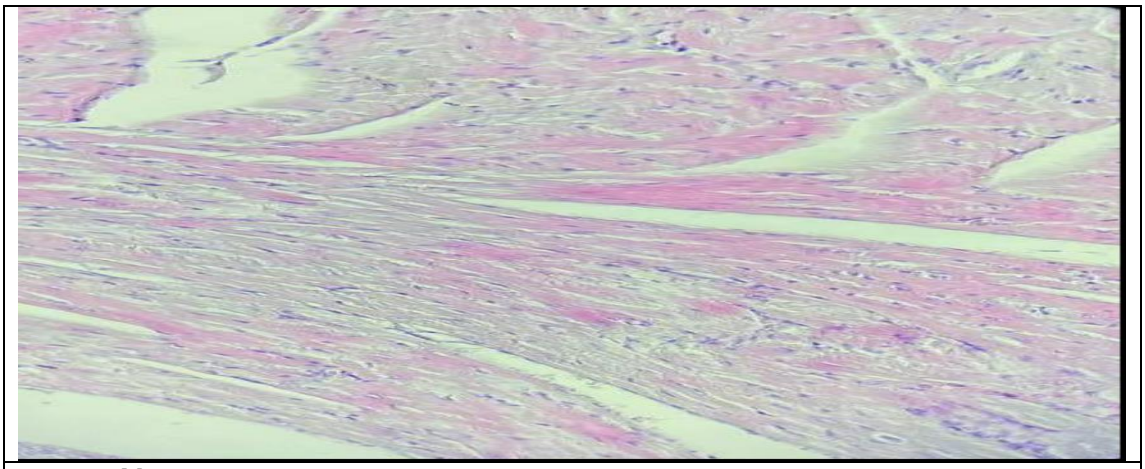
All mice in this group had normal histopathological tissue of 100 %, as shown in figure (11).

Cecal ligation and puncture (CLP) group: (B)

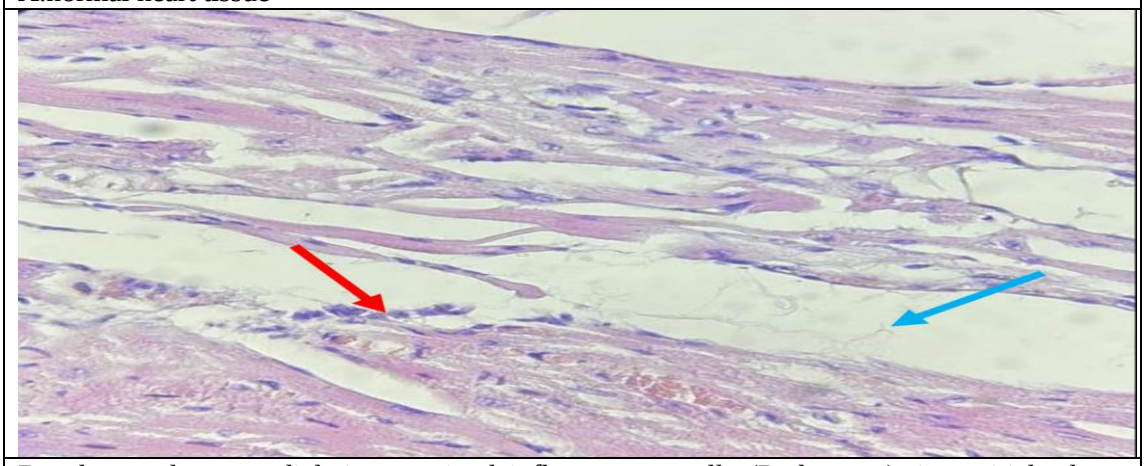
Score four injured cardiac tissue (heart tissue sections of mice in the CLP group: showed congested vessels (black arrow) & extravasation of blood cells (red arrow) as in the figure11.

Luteolin group (C)

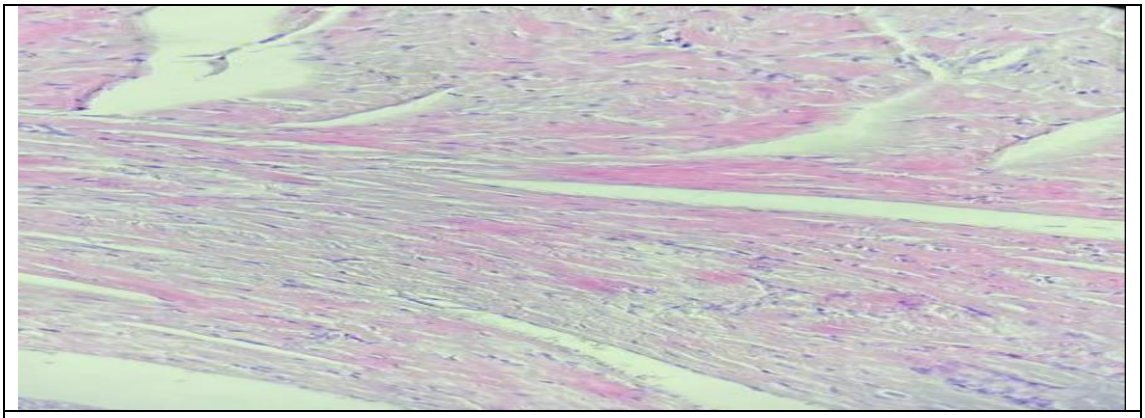
Luteolin group histological changes arranged varied from moderate to severe, Myocardial tissue sections of animal in the luteolin group: showed; contacted bands (blue arrow) & neutrophile infiltration (red arrow) as shown in figure (11).



A.normal heart tissue



B. damaged myocardial tissue, mixed inflammatory cells (Red arrow), interstitial edema (Blue arrow). This section was stained with hematoxylin and eosin (40X).



C: mild myocardial damage, and interstitial edema This section was stained with hematoxylin and eosin

Figure 11: histopathological findings of the study groups

Discussions

Organ dysfunction brought on by an unbalanced immune response to an infection is known as sepsis [22] and it is a major factor in hospitalized patients' mortality [23].

Furthermore, sepsis is known to be the primary cause of death in intensive care units. Myocardial dysfunction, also known as sepsis-induced cardiomyopathy or cardiotoxicity, is one of the major consequences linked to sepsis and significantly increases the death rate [24].

The current investigation used a cecal puncture to assess the preventive effects of luteolin in reducing cardiotoxicity during polymicrobial sepsis in mice. We demonstrated that the DMSO and CLP groups had significantly increase levels of HO-1, TNF-α, IL1B, CASPASE-11, TNFαR MPO and F2-ISOPROSTANE than the sham group. This research aligns with other earlier studies [25,26]. Evidence indicates that TNFR1 and R2 activation significantly affects immune response disruption and the ensuing sepsis-related mortality in a polymicrobial sepsis model [27]. In this investigation, the tissue level of the IL-1β was significantly higher in the CLP group than in the sham group. This finding is in agreement with a previous study that found that CLP animals had significantly higher levels of IL-1β six and twenty-four hours following CLP-induced sepsis [28,29]. In the present study, pretreatment with luteolin before poly microbial sepsis resulted in significantly lower in pro-inflammatory cytokines as compared to control group, along with IL-1β and TNFα. This result in agreement with that reported by Chen et al [30]. The current finding found a substantial decrease in TNFαR1 level in luteolin treated mice, as compared to control group. According to our current knowledge, there was a lack of available data that relating the effect of luteolin on TNFαR1 level cardiac muscles after endotoxin injury. This result might be linked to the antioxidant effect of luteolin. The current study demonstrated a significant elevation in tissue levels of Caspase11 in the CLP compared to the sham groups. This finding is in alignment with an earlier study by Mohammed *et al* [1], in the current research, administering luteolin prior to CLP led to a notable reduction in caspase-11 levels in the pretreated mice compared to control group. So luteolin demonstrated an ability to mitigate LPS-induced lethal sepsis in mice [31]. Additionally, luteolin resulted in significant lower level of

MPO in comparison with CLP group. This outcome is consistent with earlier research that showed luteolin's capacity to notably diminish neutrophil infiltration, lower the production of pro-inflammatory cytokines, and mitigate inflammation triggered by oxidative stress. The resulting cell death without infection induces a neutrophil-driven inflammatory reaction, as evidenced by the measurement of MPO content in the liver [32].

Also, luteolin causes a significant lower tissue level of oxidative stress biomarker Heme oxygenase-1 &F2-isoprostane in comparison to control group (figure 6,7). Pervious study demonstrated that Luteolin prompted an adaptive survival response in this context. This response was achieved through a mechanism that involved mitigating the oxidative cytotoxicity triggered by H2O2. This action was achieved by increasing the HO-1 and enhancing its affinity for binding to Nrf2 in the antioxidant response element. As a result of these actions, apoptosis in H9c2 cells was inhibited. These results highlighted the central role of HO-1 in mediating the cardioprotective effects of Luteolin [33].

Furthermore, there is a significant reduction in the heart level of F2-isoprostane for luteolin group as compared to CLP group. These findings suggest that luteolin have a fundamental effect in the protection of cardiac injury induced by sepsis through its anti- oxidative effect. Based on our current knowledge, there are no available data concerning the effect of luteolin on F2-isoprostane in cardiac injury induced by endotoxins. This could potentially be attributed to the antioxidative effects of luteolin.

Luteolin caused a significant increase in tissue AMPK-Nrf2 expression compared to the sepsis group (figure 9,10). This finding aligns with a study by Zhou and his coauthors which highlighted the pivotal roles played by both the AMPK and Nrf2 signaling pathways [34].

These pathways are critical in maintaining cellular energy balance and metabolic homeostasis by curbing inflammation and the production of reactive oxygen species (ROS), thus safeguarding cells during stressful conditions. Therefore, there exists a potential connection between the protective effects of luteolin and the activation of AMPK and Nrf2 signaling [34,35].

Additionally, the luteolin-treated group in this study showed a significant reduction in the degree of heart tissue damage. The luteolin group had intermediate architecture and less histological alterations, such as a moderate level of inflammation and necrotic region, when compared to the CLP group. These results are consistent with a prior investigation into the attenuation impact of luteolin against streptozotocin-induced diabetic cardiomyopathy in mice. [36].

The protection of the myocardium tissue occur from the pre-treatment with luteolin can be attributed to anti-inflammatory, antioxidant and apoptotic modulation [37] which counteract the pathological effect on the myocardium that occurs during polymicrobial sepsis induced by the CLP model. The cardioprotective action of luteolin result from inhibition of NF-κB and activation antioxidative pathway Nrf2 [36].

Conclusion

The present study contributes to the expanding evidence that luteolin exerts significant ameliorative effects in mice subjected to sepsis, primarily via anti-inflammatory, antiapoptotic and antioxidant mechanisms.

Conflicts of interest

The authors declare there are no conflicts of interest.

Acknowledgment

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Ethical considerations

The Committee for laboratory animals use & handling in University of Kufa recommended this study. The Animal Care Committee approved all animal concerns and customs. All of the mice used in this investigation were anesthetized with a combination of xylazine and ketamine before being slaughtered

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