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RESEARCH ARTICLE

CROCIN SUPPRESSES INFLAMMATORY RESPONSE IN LPS-INDUCED ACUTE LUNG INJURY (ALI) VIA REGULATION OF HMGB1/TLR4 INFLAMMATION PATHWAY

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ABSTRACT

Purpose: The most significant pathogen hypothesized to be causing the formation of Acute lung injury (ALI) in sepsis is thought to be lipopolysaccharide (LPS), a key endotoxin component of gram-negative bacteria. The main objective of this study is to determine possible anti-inflammatory effects of crocin (CRO) which has many biological properties such as anti-inflammatory, antioxidant, and anti-apoptotic in LPS-induced ALI.

Material and Methods: 40 Wistar albino rats were divided into four groups: Control (no treatment), CRO (given 50 mg/kg crocin for 9 days), LPS (given 30 mg/kg LPS at 9th day), LPS+CRO (given 50 mg/kg crocin for 9 days and 30 mg/kg LPS at 9th day). After experimental, rats were sacrificed and lungs were extracted. Histological examinations were performed in the lung tissue and the changes in the HMGB1 and TLR4 expressions were determined via immunohistochemical staining.

Results: Hemorrhage, HMGB1 and TLR4 expressions significantly increased in the LPS group. However, CRO administrations exerted a strong protective effect on the lungs in terms of these parameters in LPS+CRO group.

Conclusion: According to our results, we suggest that CRO can be considered as a protective agent against LPS induced ALI via inhibition of HMGB1/TLR4 pathway-mediated inflammatory response.

Keywords: Acute lung injury, crocin, inflammation, lipopolysaccharide

INTRODUCTION

Sepsis is an infection that causes systemic inflammatory response syndrome. It can lead to septic shock, tissue damage, multiple organ dysfunction syndrome, acute respiratory distress syndrome, and acute lung injury (ALI). Even severe sepsis can result in death (1). In sepsis, lung dysfunction is the first sign of multi-organ failure. ALI and acute respiratory distress syndrome are common complications in the intensive care unit and are

responsible for significant morbidity and mortality (2, 3). The main damage of ALI is the destruction of the pulmonary capillary endothelium and alveolar epithelium. This destruction occurs under the influence of numerous inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukins (ILs), and oxygen metabolites released by active neutrophils and macrophages (2). Lipopolysaccharide (LPS), a compound in the outer membrane of gram-negative bacteria, is a compound that impairs the immune function of many organs by triggering a biological inflammatory response (4). LPS-induced sepsis is a useful tool for studying the inflammatory process. Therefore, LPS is often applied to create an experimental model of inflammation (5, 6).

High mobility group box 1 (HMGB1) has been identified as a mediator in endotoxemia and sepsis. HMGB1 is secreted actively by innate immune cells (macrophages, monocytes) and passively by necrotic cells as a critical inflammatory mediator in cases of sepsis, shock, autoimmune diseases, and chronic inflammation. After HMGB1 is released into the extracellular environment, it binds to receptors such as Receptor for advanced glycation end-products (RAGE) and Toll-like Receptors (TLR), activating inflammatory responses (1, 7, 8). The overexpression of HMGB1 suggests that it may be an important therapeutic target in fatal systemic inflammatory diseases such as severe sepsis. Various studies have shown that the inflammatory reaction in the lung is significantly reduced by blocking HMGB1 (9, 10). TLR family consists of 10 members (TLR1-TLR10) (11). TLR4, a member of the TLR family, is a key receptor for innate immunity and cytokine release that mediates HMGB1-induced macrophage activation (8). Stimulation of TLR4 can activate the nuclear factor kappa B (NF-κB) protein. Activation of NF-κB leads to the induction of genes encoding proinflammatory cytokines such as IL-6 and TNF-α (12). LPS affects the TLR4/NF-κB signaling pathway by binding to cell surface receptors and also increases inflammation by activating inflammatory cascades in ALI (13). Highly effective pharmacotherapy is urgently needed for the treatment of acute inflammatory diseases such as ALI caused by sepsis caused by LPS.

Crosin (CRO), a bioactive natural product; It has anti-hypertensive, anti-depressant, anti-atherosclerotic, anti-platelet aggregation and nephron-protective properties as well as anti-oxidant activities and anti-inflammatory properties (14, 15). These properties of CRO make it interesting as a therapeutic agent. CRO is isolated from *Gardenia Jasminoides Ellis* and *Crocus sativus* (saffron). CRO, a water-soluble carotenoid, gives saffron its red color. (16). Saffron is grown in many parts of the world such as Iran, India, Greece, Spain, Turkey, Italy, Azerbaijan and China (17). The widespread cultivation of CRO shows that it can be easy to reach.

Elimination of inflammation that develops by activating TLR4 and NF-κB pathways by LPS or HMGB1 may lead to the prevention of possible ALI. For this purpose, reducing HMGB1 release from cells stimulated with LPS may prevent the activation of this pathway. Thus, TLR4 is not stimulated and the pathway does not become functional. Also, not activating NF-κB does not induce genes encoding proinflammatory cytokines such as IL-6 and TNF-α. Therefore, inflammation does not develop and the anti-inflammatory properties of CRO may inhibit the development of ALI. The aim of this study is to demonstrate the anti-inflammatory potential of CRO which has many biological properties on LPS-induced ALI'de. For this purpose, LPS-induced damage in the lung tissue were evaluated by histological methods and HMGB1 and TLR4 expressions were detected by immunohistochemical statining.

MATERIAL AND METHODS

Animals

This study was conceived and carried out at Erciyes University's Drug Application and Research Center in Turkey. The Experimental Animal and Local Ethics Committee of Erciyes University approved the experimental procedure used in this investigation on 02.06.2021 (Decision Number: 21/138) and 08.12.2022 (Decision Number: 22/270). Hakan Cetinsaya Experimental and Clinic Research Center, Erciyes University, provided a total of 40 male Wistar albino rats (8 weeks old, weighing 200-250 g). The rats were housed in cages in the typical order of the day, at 21°C and 12 hours of light/dark, with their water and nutrient needs met ad libitum.

Chemicals

Crocin (CRO) was purchased from Sigma-Aldrich (CAS: 42553-65-1). The purity of powdered CRO was approximately 90% according to the manufacturer's statement and it was dissolved in methanol and diluted in serum physiologic solution for the intraperitoneal injections to experimental animals

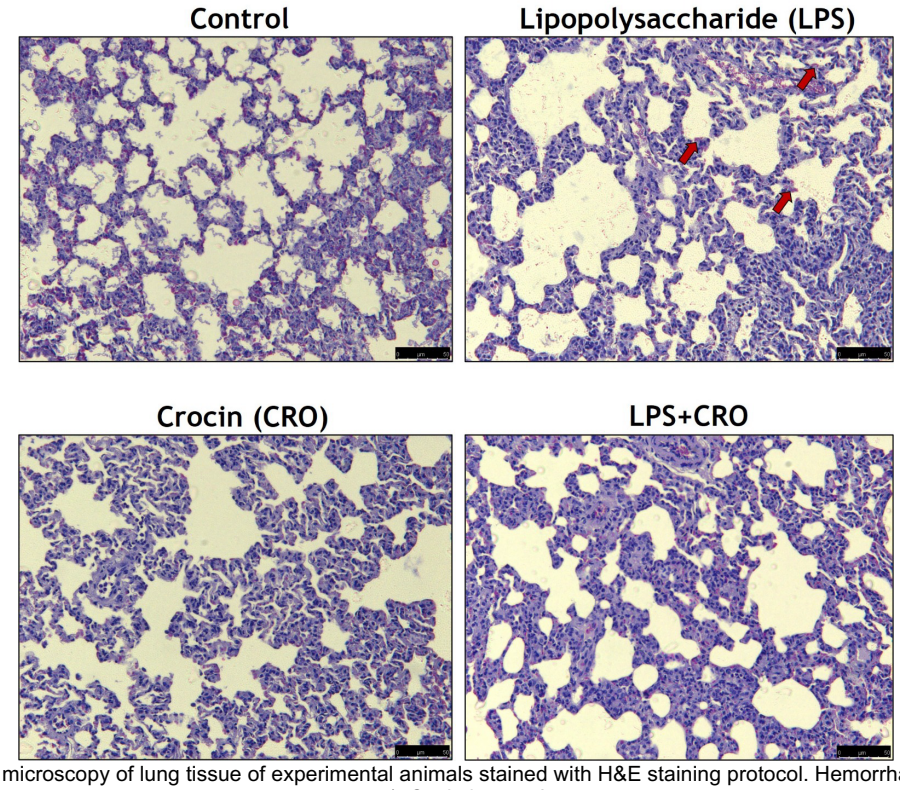

Figure 1. Light microscopy of lung tissue of experimental animals stained with H&E staining protocol. Hemorrhagic regions (red arrows). Scale bar = 50 µm

according to literatüre (18). LPS was also purchased from Sigma-Aldrich (L4130-100MG) and distilled water was used as a solvent to prepare LPS for administrations.

Experimental protocol

The sample size of this experimental study was calculated by power analysis using the G*Power v3.1 software. There was a total of 40 rats in 4 groups, 90.94% power expectation was found with 10 rats in each group. The count of animals in experimental groups was determined according to these results.

The rats were put into four groups at random, each with ten rats. The following are the groups that were formed:

1. Control group (n=10) : No treatment.
2. LPS group (n=10) : 30 mg/kg lipopolysaccharide was intraperitoneally administered to rats six hours before sacrification (18).
3. CRO group (n=10) : 50 mg/kg crocin was intraperitoneally administered to rats for 9 days (19).
4. LPS +CRO group (n=10): For 9 days, 50 mg/kg crocin was injected to rats intraperitoneally and 30 mg/kg lipopolysaccharide was administered 30 minutes after the last crocin administration.

Six hours following LPS treatment, animals were sedated with Ketamine (70 mg/bw) and Xylazine (10 mg/bw), and lung tissues were removed for histological and immunohistochemical analyses.

Histological evaluation

The lung tissues were histologically evaluated using standard histological techniques. For 24–48 hours, tissues were fixed in 10% formaldehyde, dehydrated with an alcohol series, cleared with xylene, and embedded in paraffin blocks. They were then cut into 5µm thick sections.

Hematoxylin-eosin (H&E) staining

The histological alterations in the lung tissue were determined using hematoxylin and eosin (H&E) staining (20). Images were captured and processed using a light microscope (Leica DM IL LED; Leica Microsystems, Germany). The study group looked at the structure of the lung tissue.

Immunohistochemistry

The immunohistochemistry method was applied to determine the alterations in the expression levels of HMGB1 and TLR4 antibodies as described in earlier studies of our research team. The blocks of paraffin

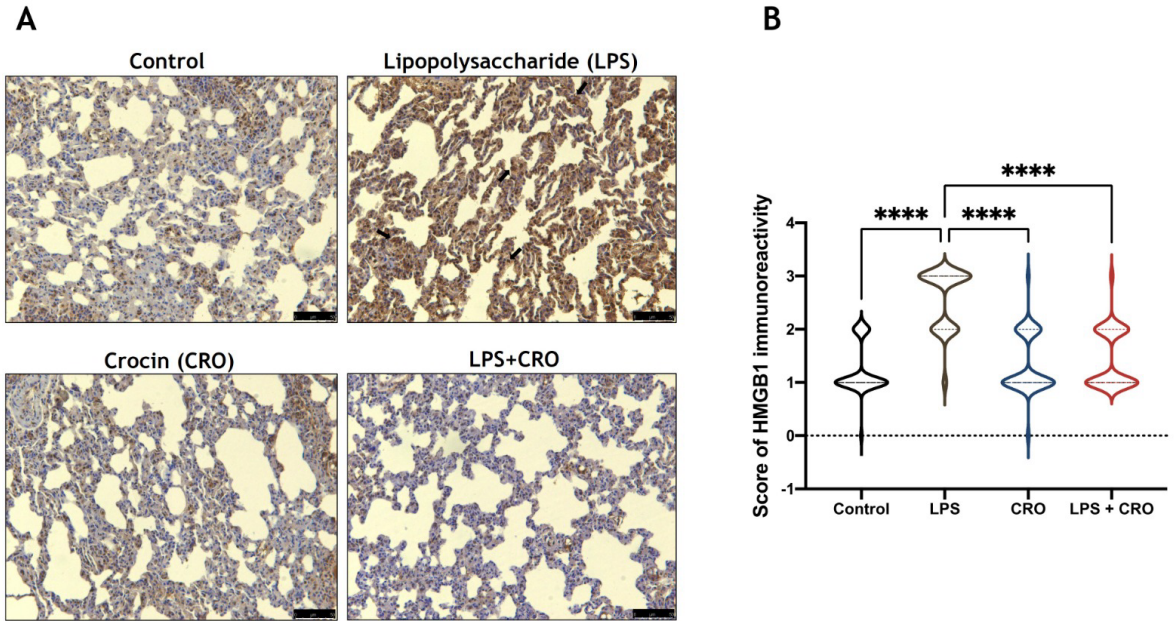

Figure 2. A. Immunohistochemical staining of HMGB1 in lung sections. Black arrows show the immunohistochemically stained areas. Scale bar: 50 µm. **B.** Statistical analysis of the immunoreactivity score among experimental groups. *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001

were cut into 5 m lengths. Xylene was used for the deparaffinization of the tissues, and they hydrated with an alcohol series. Sections were put in a sterile urine cups containing 0.01 M citrate buffer and heated in a microwave oven at 350 W for antigen retrieval. Phosphate-buffered saline (PBS) was used to wash the slices three times for five minutes each time. The slices were treated with 3 percent (w/v) H_2O_2 for 10 minutes to reduce endogenous peroxidase activity. After being rewashed three times with PBS and stored in the incubation tank for five minutes, the sections were treated with Ultra V Block solution. HMGB1 (Anti HMGB1 antibody, E-AB-70044, Elabscience, USA) and TLR4 (Anti TLR4 antibody, bs-20594R, Bioss Antibodies, USA) antibodies diluted in a 1:75 ratio were then applied to the tissues overnight at 4°C. Slices were rewashed three times with PBS the next morning before being incubated with the secondary antibody for 10 minutes (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA). The immunoreaction was amplified using streptavidin–avidin–peroxidase solution after rewashing with PBS, and the lung sections were seen with 3,3-p-diaminobenzidine tetrahydrochloride (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA) (21). The photographs were taken with a light microscope. At least ten randomly chosen fields in each slide were scored at x20 magnification. Based on histological findings, the immunoreactivity was evaluated on a scale of 0 to 3, with 0 denoting no staining and 1, 2, and 3 denoting less staining, moderate staining, and high staining, respectively (22).

Statistical analysis

All quantitative data were statistically analyzed via using GraphPad Prism v9.0 for MacOS (GraphPad Software, La Jolla, California, USA). To determine the data's normal distribution, the D'Agostino Pearson omnibus test was performed. Comparison of the quantitative variables was determined using Kruskal-Wallis and Tukey's post-hoc test. p<0.05 was used to determine statistically significant differences.

RESULTS

Histopathological findings

Lung tissue sections of the control and CRO groups showed normal histological appearance. Hemorrhagic areas were observed in the lung tissue of the LPS group. Hemorrhagic areas were observed to be reduced in the LPS+CRO group. Histological images of all groups are shown in Figure 1.

Immunohistochemical findings

HMGB1 and TLR4 expressions were observed in the lung tissue. In the LPS group, HMGB1 expressions substantially raised compared to Control and CRO groups (p<0.0001). However, In the LPS+CRO

Table 1. Scoring of experimental groups.

Groups	Control	LPS	CRO	LPS+CRO	*p*
HMGB1 immunoreactivity	1 (0-2)[a]	3 (1-3)[b]	1 (0-3)[a]	1 (1-3)[a]	0.0001
TLR4 immunoreactivity	0 (0-1)[a]	1 (0-2)[b]	0 (0-1)a	0 (0-1)[q]	0.0001

Data are expressed as median (min-max). There is no significant difference between groups with the same letter. $p<0.0001$

group, HMGB1 expressions were statistically lower when compared with those in the LPS group ($p<0.0001$) and were similar to the expression levels in the Control and CRO groups. The differences in the expression levels of HMGB1 and the statistical analysis of immunoreactivity score of experimental groups are presented in the Figure 2 and Table 1.

The expression levels of TLR4 are also significantly increased in the LPS group when compared with Control and CRO groups ($p<0.0001$). Similarly, CRO administrations were significantly preserved the lung tissue against increased TLR4 expressions in the LPS+CRO group compared to LPS group ($p<0.0001$). The differences in the expression levels of TLR4 and the statistical analysis of immunoreactivity score of experimental groups are presented in the Figure 3 and Table 1.

DISCUSSION

Sepsis, one of the deadliest diseases worldwide, often leads to multi-organ failure due to an uncontrolled inflammatory response. Sepsis is often associated with organ dysfunction induced by dysregulation of the host defense against infection. The lung is the most critical and vulnerable organ to sepsis. ALI is a common inflammatory injury caused by sepsis (23). The fact that the oxygen taken by the lungs is used in many tissues and organs shows that other organs are also affected in ALI besides the lung. Therefore, this condition, in which all organs are affected, can result in death in severe sepsis. The rate of deaths due to sepsis shock is unsettling and studies on therapeutic methods are important. Herbal-derived agents are used in public health as therapeutic agents in lung injuries as well as in many diseases. CRO, one of the active compounds of saffron, has been shown in various studies to have antioxidant, anti-cancer, anti-depressant, analgesic, anti-atherosclerosis and anti-inflammatory properties (19, 24). When the literature was carefully examined, very few studies were found showing the protective effects of CRO on tissue damage caused by LPS (19, 24-26). Therefore, in this study, we aimed to investigate the protective effect of CRO on LPS-induced acute lung injury through its potential anti-inflammatory effects.

Many studies have reported that endotoxin-induced sepsis causes severe damage to the lung tissue of experimental animals. Some studies have shown that sepsis caused by LPS applications causes many histopathological changes such as decreased pulmonary alveolar number and alveolar wall thickness, cell infiltration and hemorrhagic areas in the lungs of experimental animals (4, 27, 28). In our study, LPS application caused histopathological changes in the lung tissue, similar to previous studies. Our histological evaluations showed that this damage was in the form of hemorrhagic areas. Our histopathological scoring also supports these evaluations significantly. In the lungs of the rats to which we applied CRO preventively before LPS application, these damages were reduced and histopathological scoring decreased significantly. To understand the inflammatory mechanism of this improvement, we also evaluated HMGB1 and TLR4 immunoreactivities in lung tissue. The damage mechanism of inflammation includes binding to TLR2 and TLR4, which mediates HMGB1-dependent activation of macrophage cytokine release. Extracellular HMGB1 mediates sterile inflammation. HMGB1 binds to the receptor for TLR4, causing cells to release inflammatory cytokines. Therefore, HMGB1 and TLR4 activation is an important marker in the inflammation pathway (19, 29). Several studies have reported significantly increased expressions of HMGB1 and TLR4 in lung tissue in models of LPS-induced infection (30-32).

Treatment of LPS-induced ALI showed inhibition of inflammatory reactions by suppression of the HMGB1-mediated TLR4/NF-κB pathway (33). Previous studies have reported that CRO attenuates lipopolysaccharide-induced acute lung injury (25, 26). Also, results suggested that CRO can inhibit the NF-κB and HMGB1 inflammatory pathway activation in LPS-induced acute respiratory distress syndrome mice and LPS-stimulated human umbilical vein endothelial cells (26). In this study, unlike other studies, TLR4 expression was analysed. In our study,

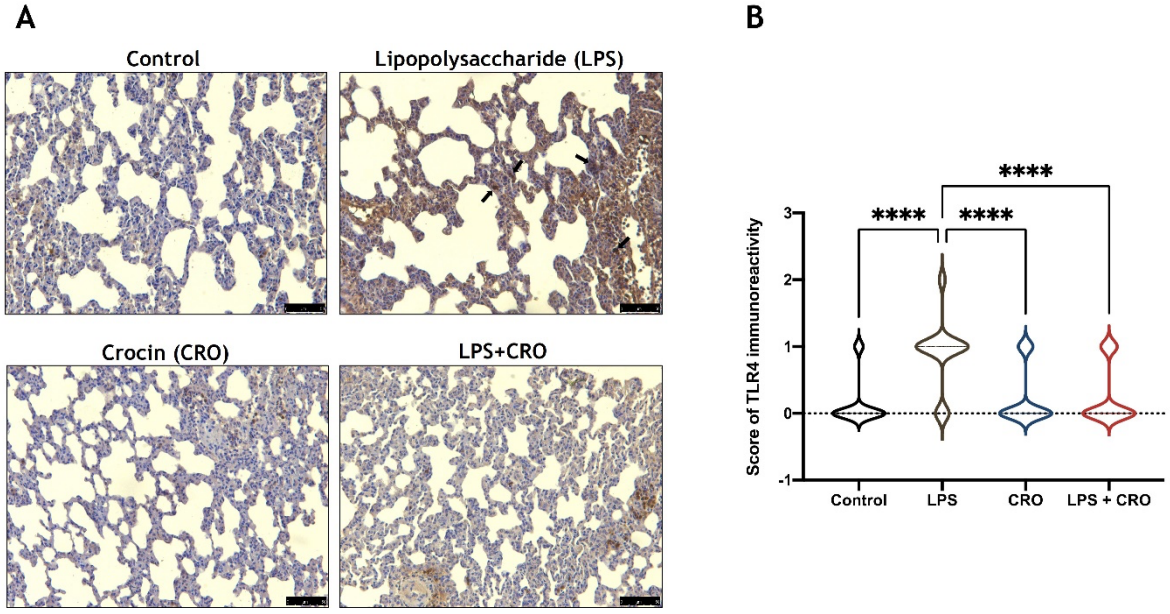

Figure 3. A. Immunohistochemical staining of TLR4 in lung sections. Black arrows show the immunohistochemically stained areas. Scale bar: 50 µm. **B.** Statistical analysis of the immunoreactivity score among experimental groups. *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$

we measured HMGB1 and TLR4 immunoreactivities to understand the mechanism of histopathological changes in lung tissue. HMGB1 and TLR4 immunoreactivities were found to be significantly higher in the lungs of animals to whom we had LPS applied. This significant value indicates that cellular damage is mediated by inflammation. It is reported that the pharmacological effects of conventional drugs and inflammatory inhibitors against HMGB1 will become new therapeutic approaches for lung diseases (34). Anti-inflammatory effects of CRO in various tissues and organs have been reported (34, 35). In our study, HMGB1 and TLR4 immunoreactivities were significantly reduced in the lungs of rats to whom CRO was administered for 9 days before LPS administration. The decrease in HMGB1 and TLR4 immunoreactivity is clear evidence that CRO has a protective effect against LPS-induced inflammation.

CONCLUSION

According to our histopathological analysis and immunohistochemical results, CRO exerted a protective effect at the dose of 50 mg/kg via attenuating histopathological changes and suppressing the inflammatory response in the lung tissue. CRO administrations can be considered as a protective treatment for the elimination of the negative effects of endotoxin-induced acute lung injury and it should not be ignored in the future clinical studies. In addition, in order to better understand the protective effect of CRO, whether it is associated with different cellular mechanisms may be a subject of research for future studies.

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Author contribution: Concept/Design: TC, ATA, EK; Data acquisition: TC, ATA, EK, SV, AT; Data analysis and interpretation: TC, ATA, EK; Drafting manuscript: TC, ATA; Critical revision of manuscript: TC, ATA, EK, AT; Final approval and accountability: TC, ATA, EK, SV, AT; Technical or material support: TC, ATA, EK, SV, AT.
Conflict of interests: The authors declare no conflict of interests.
Ethical approval: The Experimental Animal and Local Ethics Committee of Erciyes University approved the experimental procedure used in this investigation on 02.06.2021 (Decision Number: 21/138) and 08.12.2022 (Decision Number: 22/270).
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