Hesperidin Alleviates Cecal Ligation and Puncture-Induced Lung and Kidney Injuries

Hesperidin, Çekal Ligasyonu ve Delinmeye Bağlı Akciğer ve Böbrek Yaralanmalarını Hafifletir

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ABSTRACT

Objective:

The potential useful features of hesperidin (Hes) was examined against lung and kidney injuries triggered by cecal ligation and puncture (CLP) in rats in current study.

Materials and Methods:

32 Wistar Albino male rats were randomly allocated as sham, CLP, Hes 100 mg/kg and Hes 200 mg/kg groups. For the CLP process under anesthesia, the abdominal area was shaved and cleaned. The cecum was tied with a 4.0 suture and pierced by 18-gauge needle. Using this method, the experimental sepsis model was created. The lung and kidney tissues were removed at the end of the experiment. Biochemical and immunohistochemical analyzes were performed. Hes was administered by oral gavage at the doses of 100 and 200 mg/kg for 15 days.

Results:

Total oxidant status (TOS), malondialdehyde (MDA) and oxidative stress index (OSI) levels, myeloperoxidase (MPO) activity raised significantly while superoxide dismutase (SOD) and total antioxidant status (TAS) values declined in CLP group compared to sham group in lung and kidney tissues. On the contrary, SOD and TAS levels increased while MPO activity, TOS, OSI and MDA levels decreased due to Hes treatments. Caspase-3 and LC3B immunopositivity raised significantly in CLP group compared to sham group in lung and kidney tissues while decreased was observed in Hes treatment groups.

Conclusion:

In this study, in the light of our biochemical and immunohistochemical results we conclude that, Hes demonstrated an effective protection against CLP-induced lung and kidney tissue injuries in rats.

Key Words: Sepsis, Hesperidin, Lung, Kidney, Oxidative stress, Autophagy

ÖZ

Amaç:

Bu çalışmada, sıçanlarda çekal ligasyon ve delme (CLP) ile tetiklenen akciğer ve böbrek hasarlarına karşı hesperidinin (Hes) potansiyel yararlı özellikleri incelenmiştir.

Gereç ve Yöntemler:

32 adet Wistar Albino erkek rat rastgele olarak sham, CLP, Hes 100 mg/kg ve Hes 200 mg/kg

gruplarına ayrıldı. Anestezi altında CLP işlemi için karın bölgesi tıraş edilerek temizlendi. Çekum 4,0 sütür ile bağlandı ve 18' lik iğne ile delindi. Bu metod kullanılarak deneysel sepsis modeli oluşturuldu. Deneyin sonunda akciğer ve böbrek dokuları çıkarıldı. Biyokimyasal ve immünohistokimyasal analizler yapıldı. Hes, 15 gün süreyle 100 ve 200 mg/kg dozlarında oral gavaj ile uygulandı.

Bulgular:

Sham grubu CLP grubu ile kıyaslandığında akciğer ve böbrek dokularında Total oksidan durum (TOS), malondialdehit (MDA) ve oksidatif stres indeksi (OSI) düzeyleri, miyeloperoksidaz (MPO) aktivitesi anlamlı olarak artarken süperoksit dismutaz (SOD) ve total antioksidan durum (TAS) değerleri azaldı. Bunun aksine, Hes tedavilerine bağlı olarak SOD ve TAS seviyeleri artarken MPO aktivitesi, TOS, OSI ve MDA seviyeleri azalmıştır. Sham grubu CLP grubu ile kıyaslandığında akciğer ve böbrek dokularında Kaspaz-3 ve LC3B immünopozitifliği anlamlı olarak artarken, Hes tedavi gruplarında azalma gözlendi.

Sonuç:

Bu çalışmada, sunduğumuz biyokimyasal ve immünohistokimyasal bulgularımız ışığında, Hes' in sıçanlarda CLP' ye bağlı akciğer ve böbrek dokusu hasarlarına karşı etkili bir koruma sağladığı belirlenmiştir.

Anahtar Sözcükler: Sepsis, Hesperidin, Akciğer, Böbrek, Oksidatif stres, Otofaji

INTRODUCTION

Sepsis is a complicated syndrome that occurs in case of systemic inflammatory response syndrome (SIRS). SIRS results from infection which is mediated by endogenous mediators affecting all organs and systems (1). Sepsis leads to respiratory, renal, hepatic, cardiovascular and endocrine organ dysfunctions as a result of many changes such as increased microvascular permeability, acute lung injury, coagulation abnormalities, hypovolemia, decreased myocardial contractility, hypoxia, decreased systemic vascular resistance and hyperglycemia (2). Lungs are believed to be the first and mostly affected organ due to intraabdominal sepsis (3). SIRS can develop for many reasons. Localized or widespread infections, trauma, burns or acute pancreatitis may be the cause of SIRS (4). The most important source of free oxygen radicals (ROS) for aerobic organisms is molecular oxygen. 98% of molecular oxygen is normally converted to water by cytochrome oxidase enzyme, while the rest is converted into the reactive toxic products of reduction. ROS also occurs due to enzymatic oxidation of arachidonic acid, as an intermediate product of catalytic cycles of enzymes. We can expect ROS formation due to the oxidation of unsaturated fatty acids or during phagocytosis function of phagocytes (5). In pathological processes such as ischemia and sepsis, the equilibrium between oxidants and antioxidants is disrupted and this results in oxidative stress which reveals the harmful effects of oxidants (6). Oxidative stress is involved in the immune system's response to systemic damage, both with its extracellular effects and as an intracellular signal. In sepsis, ROS plays a key role in the pathogenesis of hemodynamic disorder and organ failure. It induces cytotoxicity in organs and causes changes in cell signal pathways (7).

Hesperidin (Hes), a flavonoid; consists of flavanone hesperitin and disaccharide rutinoz. Hes is the most common flavonoid in orange and lemon (8). It has anti-inflammatory, antioxidant, anti-allergic, hypolipidemic, anti-carcinogenic and vascular protective effects (9, 10). Hes has been shown to affect the histamine release and arachidonic acid metabolism (7). The effect of the Hes to destroy free oxygen radicals has been reported in the literature. It also inhibits the effects of pro-inflammatory mediators (11).

Here, we investigated the possible benefical effects of Hes against lung and kidney injuries induced by CLP model which is similar to clinical sepsis with metabolic and hemodynamic properties.

MATERIALS and METHODS

Animals and Ethical Approval

Experimental process of the study was performed at Experimental Animals Research and Application Center of Atatürk University. The present study was admitted by Atatürk University Experimental Animal Ethics Committee (Protocol Number:25.01.2018/1). 32 Wistar type Albino male rats weighing 270-280 g, were acquired from the same center. Rats were held in cages with appropriate conditions including humidity of 55±5%, temperature of 22±2 °C under a 12 h light /12 h dark cycle. Rats were supplied with standard rat feed and drinking water. All animals were deprived of food 12 hours prior to the experiment, but were allowed to drink water.

Experimental Process, Drugs and Groups

Rats were fixed in supine position. The abdominal region of the animals were shaved and disinfected via 10% povidone iodine. The experimental process was performed under anesthesia. Thiopental sodium (50 mg/kg, Ulagay, İstanbul, Turkey) was preferred for the anesthesia (12). Hes solution was prepared in 0.5% CMC. Hesperidin (98%) and sodium carboxymethyl cellulose (CMC) was provided by Sigma-Aldrich Co. USA.

Rats were weighed and classified as 4 groups. The groups were designed as follows;

Sham group (n=8): In the abdominal midline of the rats, a 1-2 cm incision was made and closed back. After 16-18 hours (sepsis time), tissues of lungs and kidneys were excised.

CLP group (n=8): Following the same incision in sham group, the abdominal muscles and peritoneum were crossed and the cecum was removed. It was tied with a 4.0 suture with a size of 2 cm at the distal part of cecum. In addition, 4 holes were drilled from this distal cecum region with the help of an 18 gauge needle. Afterwards, the cecum and intestine sections were placed back into abdomen. 2 ml of saline was emptied into the abdomen and the incision line was sutured. CLP method was selected with the reference of a previous experimental study (13).

Hes 100 mg/kg group (n=8): 100 mg/kg Hes was adminis-

tered once a day for 15 days by oral gavage. After the application of last dose of Hes, CLP induction was carried out as described in group II.

Hes 200 mg/kg group (n=8): 200 mg/kg of Hes was administered once daily for 15 days with oral gavage and all procedures in the group II were repeated.

After 16-18 hours, these rats were sacrificed, the lung and kidney tissues were collected.

Biochemical Measurements

Tissue samples, each weighing 100 mg, were homogenized by 2 mL of phosphate buffer solution (PBS) and centrifuged at 5000 rpm at +4 °C for 20 minutes. The supernatants were kept at -80 °C in eppendorf tubes. The values of total oxidant status (TOS) and total antioxidant status (TAS) were determined using ELISA kits (Rel Assay Diagnostics). The rate of TOS to TAS was accepted as oxidative stress index (OSI). The measurement of malondialdehyde (MDA) (14), myeloperoxidase (MPO) (15) and superoxide dismutase (SOD) (16) levels were carried out as described in previous studies.

Immunohistochemical (IHC) Staining

After the kidney and lung tissues were held in neutral formaldehyde solution for 24 hours, formaldehyde was removed by washing with tap water. Tissues were routinely blocked through alcohol-xylol and blocked in paraffin. Following the tissue deparaffinization, they were taken on the polylysine coated slide and left for 10 minutes in 3% H2O2 and washed in phosphate buffered solution (PBS) to passivate the activity of endogenous peroxidase. Then, they were held in antigen retrieval solution at 500w for 10 minutes and washed in PBS to reveal antigens in the tissues. To prevent nonspecific binding, protein block solution was added and washed in PBS. Light chain 3 (LC3B) (Abcam, Cat. No: ab48394 Dilution: 1/200) and cleaved caspase-3 (Novus Biological, Cat. No: NB600-1235, Dilution: 1/100) were applied as primary antibody to PBS-washed sections. After all, the procedure specified by Expose mouse and rabbit specific HRP/DAB detection IHC kit (Abcam: ab80436) was performed. 3.3 'diaminobenzidine chromogen was used and hematoxylin was preferred for contrast staining. Positive cells were investigated under a light microscope at 20x magnification.

Statistical Analyses

Firstly, one-way ANOVA test was chosen for biochemical data and then Tukey HSD test was preferred for multiple comparisons. The results were given as Mean±Standard Deviation (SD). A p value below 0.05 was considered statistically significan.

RESULTS

Lung Tissue Biochemical Results

The changes in TAS, TOS, OSI parameters were demonstrated in Table I. SOD, MPO, MDA levels were presented in Figure 1. TAS level decreased in CLP group compared to sham group and increased in both Hes treatment groups compared to CLP group ($p<0.05$). TOS, MPO, MDA and OSI levels were elevated in CLP group compared to sham group and diminished in both Hes treatment groups compared to CLP group ($p<0.05$). SOD enzyme levels did not differ among the groups ($p>0.05$).

Table I: Effects of Hes treatment on biochemical parameters in CLP-induced lung injury.

Experimental Groups (n=8)	TAS (mmol/L)	TOS (μmol/L)	OSI (arbitrary unit)
Sham	0.79 ± 0.14	8.75 ± 1.00	1.11 ± 0.18
CLP	0.23 ± 0.09^a	10.36 ± 0.81^a	5.22 ± 2.15^a
Hes 100 mg/kg	0.65 ± 0.20^b	8.50 ± 0.85^b	1.42 ± 0.47^b
Hes 200 mg/kg	0.73 ± 0.08^b	8.41 ± 0.37^b	1.04 ± 0.42^b

[a]p < 0.05 compared to sham group. [b]p< 0.05 compared to CLP group.

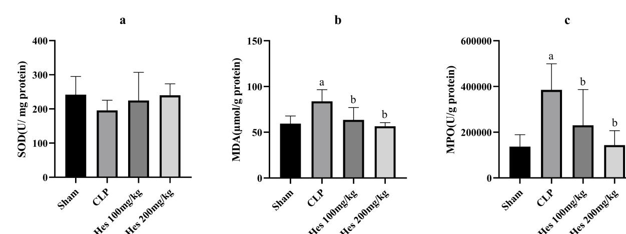

Figure 1: Effect of Hes on the levels of (a) SOD, (b) MDA and (c) MPO in CLP-stimulated lung tissues. ap<0.05 compared to sham group and bp<0.05 compared to CLP group. Data were expressed as Mean±SD.

Kidney Tissue Biochemical Results

The changes in TAS, TOS and OSI parameters (Table II) and SOD, MPO and MDA parameters (Figure 2) were presented. No significant difference among the groups was detectedfor the TAS levels ($p>0.05$). TOS, OSI and MDA levels raised in CLP group compared to sham group and declined in HES treatment groups compared to CLP group ($p<0.05$). SOD enzyme levels decreased in CLP group compared to sham group ($p<0.05$). SOD level increased in high dose Hes treatment group compared to CLP group ($p<0.05$). MPO activity did not display any difference among the groups ($p>0.05$).

Table II: Effects of Hes treatment on biochemical parameters in CLP-induced kidney injury.

Experimental Groups (n=8)	TAS (mmol/L)	TOS (μmol/L)	OSI (arbitrary unit)
Sham	1.67 ± 0.38	6.80 ± 0.96	0.42 ± 0.10
CLP	1.35 ± 0.28	8.35 ± 0.57^a	0.65 ± 0.18^a
Hes 100 mg/kg	1.63 ± 0.22	7.10 ± 0.94^b	0.44 ± 0.10^b
Hes 200 mg/kg	1.57 ± 0.26	6.74 ± 0.73^b	0.41 ± 0.05^b

[a]p<0.05 compared to sham group. [b]p<0.05 compared to CLP group.

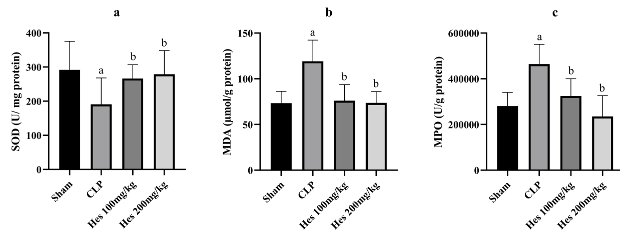

Figure 2: Effect of Hes on the levels of (a) SOD, (b) MDA and (c) MPO in CLP-stimulated kidney tissues. ap<0.05 compared to sham group and bp<0.05 compared to CLP group. Data were expressed as Mean±SD.

IHC Results

Caspase-3 and LC3B IHC staining in lung tissues were presented in figure 3. For the lung tissues, there was no caspase-3 immunopositivity in sham group while it was intense in CLP group. A decrease in caspase-3 immunopositivity was observed in Hes 100 mg/kg dose group. In Hes 200 mg/kg dose group, caspase-3 immunopositivity was found quite mildly. There was no immunopositivity in sham group for the LC3B staining. In Hes 100 mg/kg dose group, a decrease in LC3B immunopositivity was observed and it was quite mild in Hes 200 mg/kg dose group.

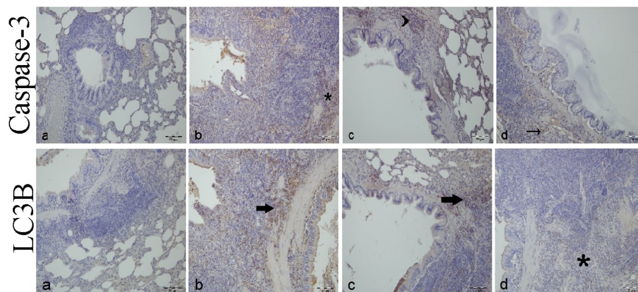

Figure 3: Presentation of Caspase-3 and LC3B IHC staining in lung tissues a) Sham group b) CLP group, intense immunopositivity in BALT (star or arrow) c) Hes 100 mg/kg, moderate immunopositivity in BALT (arrow or arrow head) d) Hes 200 mg/kg group, mild immunopositivity in BALT (star or arrow).

Caspase-3 and LC3B IHC staining in kidney tissues are presented in Figure 4. Caspase-3 immunopositivity was not seen in sham group. It was observed intensively in tubules and glomerulus samples of CLP group. In Hes 100 mg/kg dose group, caspase-3 immunopositivity was at medium severity in tubules and it was mildly present in tubules of Hes 200 mg/kg dose group. There was no immunopositivity in sham group for the LC3B staining. On the other side, LC3B immunopositivity was intense in both CLP and Hes 100 mg/kg dose groups. In Hes 200 mg/kg dose group, a decrease in LC3B immunopositivity was observed.

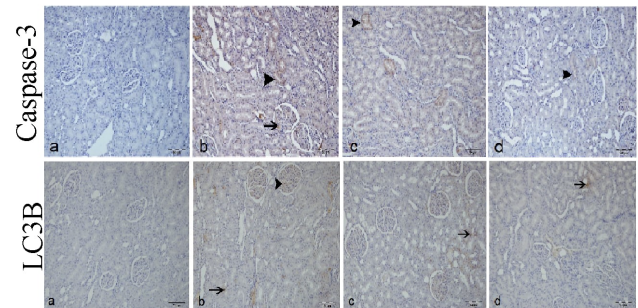

Figure 4: Presentation of Caspase-3 and LC3B IHC staining in kidney tissues a) Sham group b) CLP group, glomerulus (arrow) and tubules (arrow head)display intense immunopositivity c) Hes 100 mg/kg, moderate immunopositivity in tubules (arrow head) d) Hes 200 mg/kg group; mild immunopositivity in tubules (arrow head).

DISCUSSION

Sepsis is the widespread inflammatory response which can occur in both primary focus of infection and remote organs (17). It is characterized by infection and inflammation which causes mortality (18). Surviving ratio of septic patients increases with care (19). Sepsis is an important and extensive clinical condition demonstrating morbidity and mortality. 20-30% of one million sepsis cases results in death in North America annually. Sepsis is the most common death cause in hospitalized patients (20-22). It mostly results from bacterial infections. It causes end organ failure. Infection induces SIRS which causes multiple organ failure and immunological abnormalities (23, 24). CLP model has been used to be able to solve the mechanisms of sepsis (25, 26). It is widely used among the many murine models of sepsis (27, 28). In CLP experimental model, intestinal flora is used to form peritonitis and this resembles bowel perforation. Sepsis duration and range of bacterial flora are quite similar with clinical sepsis cases (29, 30).

Free radicals emerge as the natural product of physiological activity in the body and they are balanced by the oxidant-antioxidant mechanisms. In cases where excessive production of free radicals occurs or antioxidant defense mechanisms remain incapable, oxidative stress occurs. Due to oxidative stress, cell damage can occur as a result of free radicals reacting with cell membrane lipids, DNA, carbohydrates and proteins (31). Antioxidants reduce the damage caused by radicals, suppress radical formation mechanisms, neutralize the produced radicals, stop chain reactions that cause radical production including lipid peroxidation and ultimately provide cell defense (32). Even though several studies have been performed (13, 33-35), the pathophysiology of sepsis in humans remains unclear (36, 37). Clinical and experimental studies support the benefits of antioxidants against sepsis including lung injury as remote organ (38, 39). Antioxidants may alleviate sepsis related inflammation and tissue injury by means of free radical scavenging and enhancing antioxidant

defense system (40, 41).

Free radicals lead to lipid peroxidation which reduces membrane potential and results in cell injury. MDA is a lipid peroxidation product and also causes serious cell damage (42). Therefore, ROS and MDA are considered as biomarkers for the detection of oxidative stress (43). Elevation was observed in MDA levels after lipid peroxidation in CLP-induced rat sepsis models (44, 45). MPO enzyme is considered as an indicator for neutrophil infiltration (46). During inflammation, neutrophils release excessive amounts of MPO (47). SOD is the primary antioxidant associated with scavenging free radicals (48).

High levels of ROS in the cells lead to the stimulation of the apoptosis, while the low levels act as a signaling molecule that regulates cell growth and survival (49). When the ROS production is inducted, it results in pro-apoptotic gene upregulation, caspase activation and apoptotic cell death (50). The caspases are triggered by signals that activate apoptosis and actively take part in apoptosis pathways (51). Caspase 3, 6 and 7 are responsible for breaking down of cells (52). In previous studies using an experimental sepsis model, it was found that especially MDA levels increased significantly in sepsis, but the antioxidant defense was insufficient (44, 45, 53). The results of the present study are consistent with the findings of previous studies.

Autophagy involves autophagosome formation that ingests dysfunctional or damaged organelles and proteins. Actually, autophagy is necessary for development and differentiation as well as cellular maintenance (54-56). Although autophagy maintains cellular homeostasis, it also can be protective or harmful due to the conditions (57). LC3B is a cellular indicator of autophagy (58). In the beginning of autophagy, phagophore occurs. Phagophore is a sort of membrane where LC3-I and lipids are attached (59, 60). LC3-II accumulation is evaluated as a marker of damaged autophagy (61). In a CLP-induced experimental sepsis model, the level of LC3-II in the heart tissues were significantly increased (62, 63). In accordance with the results of previous studies, current study shows that LC3B immunopositivity is intense in CLP and decreases with Hes administration.

CONCLUSION

In this study, it has been shown that treatment with Hes reduces lung and kidney injuries in experimental animals exposed to CLP-induced sepsis model. Further studies would be helpful to explain the possible protective mechanisms in lung and kidney injuries induced by sepsis.

Conflict of interest

None.

Data availability statement

None.

Ethics Committee Approval

The present study was admitted by Atatürk University Experimental Animal Ethics Committee (Protocol Number:25.01.2018/1).

Financial Disclosure

No support was received from any institution in the realization of this study. The necessary resources for the study were provided by the authors. There is no financial conflict between the authors.

Author Contributions

Güzel Erdoğan D, Tanyeli A, Ekinci Akdemir FN, Güler MC, and Eraslan E contributed in the planning of design and animal experimental process, literature review, critical language revision and writing of this study. Çomakli S performed histopathological and immunohistochemical evaluations. Polat E carried out the biochemical measurements and statistical analyses.

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