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Cecal Ligation and Puncture-Induced Sepsis Model in Rats

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Abstract: Sepsis is a worldwide health problem with a high mortality rate. It may result from various conditions, including trauma, surgery, and infection. Kidneys and lungs rank among the sepsis-induced injured organs. Cecal ligation and puncture (CLP) is similar to clinical sepsis and is preferred to imitate clinical diverticulitis or appendicitis perforations. Here, we designed a CLP model as a prestudy for further protective agent studies against CLP-induced sepsis. We aimed to find biochemical and histological results compatible with previous experimental CLP models in the literature. We used 20 female Wistar Albino rats. We created two randomized groups, a sham group, and a CLP group (n=10). We measured interleukin 1-beta (IL-1β), IL-6, and IL-10 cytokine levels. We assessed myeloperoxidase (MPO), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) levels in renal and lung tissues. We also examined the tissue samples for histopathological and immunohistochemical evaluation. Cytokine levels and oxidant parameters were increased, and antioxidant parameters decreased in the CLP group as an indicator of a strong immune response due to sepsis. Histopathological and immunohistochemical findings supported the biochemical findings on behalf of sepsis. The results demonstrated a meaningful sepsis picture indicating a successful CLP model, which we may prefer to use in further CLP-induced sepsis models.

Keywords: Cecal ligation and Puncture, Rat, Sepsis.

Sıçanlarda Çekal Ligasyon ve Delme Kaynaklı Sepsis Modeli

Öz: Sepsis, yüksek ölüm oranına sahip dünya çapında bir sağlık sorunudur. Travma, cerrahi ve enfeksiyon gibi çeşitli durumlardan kaynaklanabilir. Böbrek ve akciğerler sepsise bağlı hasarlanan organlar arasında yer alır. Çekal ligasyon ve delme (CLP) klinik sepsise benzerdir. Klinik divertikülit veya apandisit perforasyonlarını taklit etmek için tercih edilir. Bu çalışmayı, ileride gerçekleştirmeyi planladığımız CLP ile indüklenen sepsise karşı koruyucu ajan çalışmaları için bir ön çalışma olarak tasarladık. Literatürde bulunan deneysel CLP modelleriyle uyumlu biyokimyasal ve histolojik sonuçları bulmayı hedefledik. Çalışmada 20 adet dişi Wistar Albino sıçan kullandık. Rastgele iki grup oluşturduk; sham grubu ve CLP grubu (n=10). İnterlökin 1-beta (IL-1β), IL-6 ve IL-10 sitokin seviyelerini ölçtük. Böbrek ve akciğer dokularında miyeloperoksidaz (MPO), süperoksit dismutaz (SOD), malondialdehit (MDA) ve glutatyon (GSH) düzeylerini belirledik. Ayrıca doku örneklerini histopatolojik ve immünohistokimyasal değerlendirme için inceledik. CLP grubunda sepsise bağlı güçlü bir immün yanıtın göstergesi olarak sitokin seviyeleri ve oksidan parametreleri artarken antioksidan parametreler azaldı. Ayrıca, histopatolojik ve immünohistokimyasal bulgular biyokimyasal bulguları sepsis lehine destekledi. Elde ettiğimiz sonuçlar ileride CLP ile indüklenen sepsis modeli olarak kullanabileceğimiz başarılı bir CLP modelini gösteren anlamlı bir sepsis tablosu oluşturdu.

Anahtar Kelimeler: Çekal ligasyon ve Delme, Sepsis, Sıçan.

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INTRODUCTION

Sepsis is a serious health condition with a high mortality rate (Dellinger et al., 2013). It influences more than 19 million people worldwide annually (Prescott & Angus, 2018). Major surgery, burns, shock, and many health conditions may lead to sepsis (Rhodes et al., 2017). Sepsis is an infection-related systematic inflammatory disease (Cheng et al., 2017). There is a host response failure (Benz et al., 2016) and a dysfunctional inflammatory host reaction (Cecconi et al., 2018) against infections. Severe inflammation leads to multiple organ dysfunctions (Seymour et al., 2016). Kidneys and lungs rank among the sepsis-induced injured organs (Lan et al., 2017). Certain animal studies have examined sepsis-induced acute lung injury (ALI) (Sadowitz et al., 2011; Xiao et al., 2014). ALI occurs in various severe sepsis cases (Zimmerman et al., 2009). Sepsis-induced lung injury plays a role in inflammatory cascade via neutrophil activation (Park et al., 2019).

High neutrophil levels enhance reactive oxygen species (ROS) production which causes oxidative stress injury (Bi et al., 2017). Oxidative stress leads to malondialdehyde (MDA) generation (Till et al., 1985). MDA levels raise in cecal ligation and puncture (CLP)-induced septic lung tissues (Y. Wang et al., 2018). Neutrophils secrete myeloperoxidase (MPO) (Srivastava et al., 2010) that is used to assess the neutrophil accumulation in inflamed tissues (Lingaraju et al., 2015). Superoxide dismutase (SOD) is a primary defense preventing oxidative stress. CLP elevated hepatic MPO activity and decreased hepatic SOD activity in a previous study (Makled et al., 2016). Glutathione (GSH) plays a key role in oxidative stress (Dröge, 2002). Oxidative damage decreases GSH levels (Seibt et al., 2019).

High proinflammatory cytokine levels increase the mortality rate during sepsis (Mikkelsen et al., 2013). Bacterial toxins initiate several pro-inflammatory cytokine productions such as interleukin 1 (IL-1) and IL-6 which are vital for sepsis (Galanos & Freudenberg, 1993). Sepsis-induced ALI elevates pro-inflammatory cytokines, particularly IL-1β and IL-6 levels (Polat et al., 2013). In addition to the pro-inflammatory response, anti-inflammatory mechanisms also occur during sepsis (Potjo et al., 2019). IL-10 levels were correlated with IL-6 levels in a previous CLP model (Zhang et al., 2021).

Various sepsis models, including CLP have been examined for more than 30 years on animals (Schabbauer, 2012). CLP is a similar model to clinical sepsis and is preferred to imitate clinical diverticulitis or appendicitis perforations (Bastarache & Matthay, 2013; Song et al., 2013). Here, we designed a CLP model as a prestudy for further protective agent studies against CLP-induced sepsis. We aimed to find biochemical and histological results compatible with previous experimental CLP models in the literature. Thus, we hope to create more comprehensive and multidisciplinary CLP model research via experiencing CLP model creation.

MATERIALS and METHODS

Ethical Approval and Experimental Animals

Atatürk University Experimental Animal Ethics Committee confirmed the experiment (11.06.2020-95). Atatürk University Experimental Animal Research and Application Center procured the experimental animals and surgical room for the experimental procedure. We used 20 female Wistar Albino rats (200 gr-250 gr). We housed the animals under laboratory conditions at 22-250°C with a 12-/12-h light/dark cycle. We granted food and water access ad libitum.

Chemicals, Experimental Groups, and CLP Model

We anesthetized and immobilized the rats in the horizontal position. Then, we shaved and disinfected the abdominal regions. We used 15 mg/kg intraperitoneal (i.p.) xylazine hydrochloride (Rompun®, Bayer, Istanbul), 100 mg/kg i.p. ketamine (Ketalar®, Pfizer, Istanbul) for anesthesia (Bayraktutan et al., 2021), and 10% povidone-iodine solution (Batticon; Adeka,) for disinfection. We

created two randomized groups, a sham group, and a CLP group.

Sham group (n=10): We incised longitudinally at 2-2.5 cm, along the ventral line below the xiphoid. We only opened the abdominal cavities of the animals and closed back.

CLP group (n=10) = We established a CLP model modified from previous studies (Daniel Rittirsch et al., 2009; Linlin Song et al., 2018). Following the abdominal incision, we reached the ventral cavity. We identified and exteriorized the cecum. We dissected the mesentery of the cecum carefully avoiding an injury in the cecal branch of the ileocecal artery.

In addition to sepsis duration and needle size, cecum ligation length also determines the severity of the CLP model (Ruiz et al., 2016). We performed a medium ligation to induce a mid-grade sepsis. The distance between the distal pole and ligation, and the distance from the ligation to the basis of the cecum were nearly the same. We ligated distal cecum lower than the ileocecal valve level to prevent intestinal obstruction.

We punctured the cecum using an 18-gauge needle to take a single pass through the cecum. We removed the needle and extruded a small amount of fetal content to make sure of the holes. We replaced the cecum into the abdominal cavity and closed the incision with 3.0 silk suture. We injected normal saline (0.9% NaCl, 50 mL/1 kg, 370°C) subcutaneously as postoperative fluid resuscitation. We put back the rats in the cages following the experimental process. The animals were free to access food and water.

A mid-grade CLP-induced sepsis model (18-gauge needle, medium ligation) occurs at approximately 16 hours (Hubbard et al., 2005). Thereby, after 16 hours, we sacrificed the rats under high-dose anesthesia. We collected blood, lung, and renal tissue samples for biochemical and histological analysis.

Biochemical Analysis

We added 10% phosphate buffer solution (PBS) to lung and kidney tissues and homogenized them. Following the homogenization, we centrifugated the samples for 30 minutes at 5000 rpm. We used the supernatant for SOD, GSH, MDA, and MPx determination. We measured MDA, MPx, SOD, and GSH levels according to the methods mentioned in previous studies (Bradley et al., 1982; Hu, 1994; Ohkawa et al., 1979; Sun et al., 1988). We used IL-1β, IL-6, and IL-10 rat-specific ELISA kits (Elabscience, Wuhan, China) to evaluate the blood cytokine levels according to the protocols of the kits.

Histopathological Examination

At the end of the study, the rats were euthanized, and their kidney and lung tissues were placed in 10% buffered formaldehyde solution. After the fixation phase, the samples were shrunk, subjected to routine follow-up processes, and embedded in paraffin. Sections of 5 µm were taken on a normal slide and stained with hematoxylin-eosin to examine for histopathological changes. Lung sections were examined for the thickness of the alveolar wall, and the kidney tissue for necrosis, degeneration, and glomerular damage under the light microscope.

Immunohistochemical (IHC) Staining

5 µm sections from the tissue samples in the paraffin block were taken on a polylysine slide, and then the tissues were deparaffinized. To inactivate endogenous peroxidase activity, the tissues were kept in 3% hydrogen peroxide (H2O2) for 10 minutes. Then, boiling was performed in the antigen retrieval solution at 800w for 10 minutes to reveal the antigens in the tissues. Protein block solution was added to the tissues to prevent nonspecific binding. Sections washed with PBS were treated with Caspase-3 (Santa Cruz, Cat. No: sc-65497, Dilution:1/100) as the primary antibody. Then, the procedure specified by the Expose mouse and rabbit specific HRP/DAB detection IHC kit (abcam:

ab236466) was followed. Antigen-antibody binding to 3,3' diaminobenzidine using chromogen was made visible. Tissues counterstained with hematoxylin were examined for positive staining under the light microscope at 20x magnification.

Statistical Analysis

The IL-1β, IL-6, and IL-10 results were evaluated via IBM SPSS 20.0 Package Program. The normality test was performed and then the One-Way ANOVA test was applied. Following these tests, the data were evaluated according to Duncan, one of the post-hoc tests, since it was parametrically appropriate. The data were represented as mean ± standard deviation (SD). In the analyses, $p<0.05$ values were considered statistically significant.

RESULTS

Biochemical Results

Figure 1 represents the blood cytokine levels. IL-1β, IL-6, and IL-10 elevated significantly in the CLP group compared to the sham group (figure 1a,1b,1c $p<0.05$). Figure 2 demonstrates the oxidant and antioxidant parameters in the renal tissue samples. SOD and GSH diminished in the CLP group compared to the sham group (figure 2a, figure 2b $p<0.05$). MDA and MPx were raised in the CLP group compared to sham group (figure 2c, figure 2d $p<0.05$). Figure 3 shows the oxidant and antioxidant parameters in the lung tissue samples. SOD and GSH decreased in the CLP group compared to the sham group (figure 3a, figure 3b $p<0.05$). MDA and MPx elevated in CLP group compared to sham group (figure 3c, figure 3d $p<0.05$).

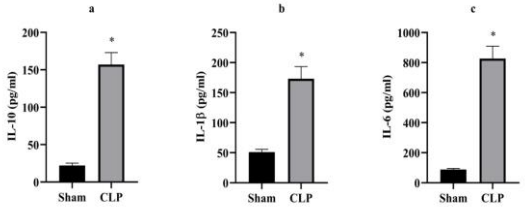

Figure 1. Cytokine levels of sham and CLP groups (* $p<0.05$).

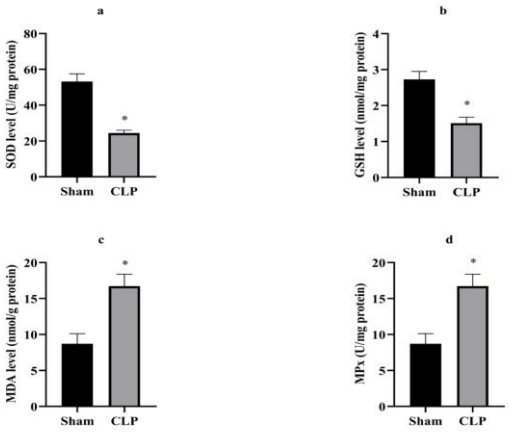

Figure 2. Antioxidant and oxidant parameters of renal tissue samples (* $p<0.05$)

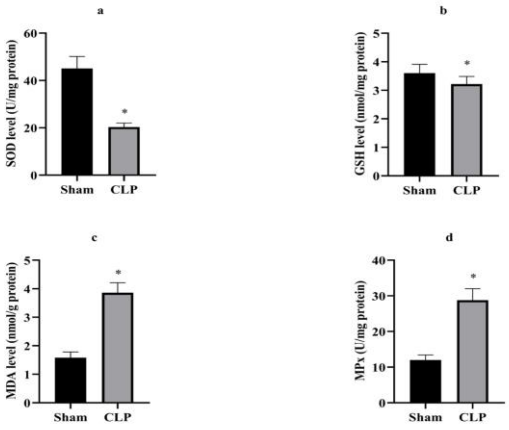

Figure 3. Antioxidant and oxidant parameters of lung tissue samples (* $p<0.05$).

Histopathological Results

In the histopathological examination of the kidney tissue, normal glomerulus and tubular epithelium was observed in the sham group (figure 4a). In the CLP group, atrophy in the glomerulus and necrotic changes occurred in the tubular epithelium (figure 4b).

In the histopathological examination of the lung tissue, there was a normal lung histological appearance in the sham group. Alveolar wall thickness was normal (figure 4c). In the CLP group, thickening was detected in the interalveolar space due to mononuclear cell infiltration (figure 4d).

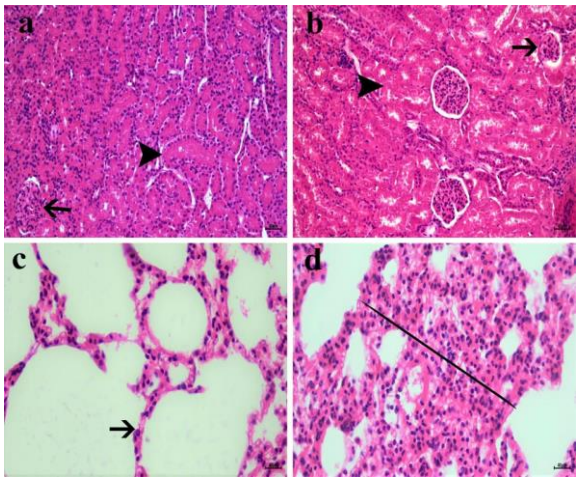

Figure 4. a) Sham group; normal kidney histological appearance. Normal glomerular (arrow) and tubular structures (arrowhead), b) CLP group; necrosis of the tubular epithelium (arrowhead) and atrophy of the glomerulus (arrow), c) Sham group; normal lung alveolar structure (arrow), d) CLP group; severe thickening of the alveolar wall. HEx20.

Immunohistochemical Results

There was no Caspase-3 immunopositivity in the kidney and lung tissue samples of the sham group (Figure 5a, Figure 5c). Intense Caspase-3 immunopositivity was detected in the kidney and lung tissues of the CLP group (Figure 5b, 5d). The immunopositivity occurred in the tubular epithelium and glomerular tuft in the kidney tissue, and it was observed in the inflammatory cells of the alveolar space in the lung tissue.

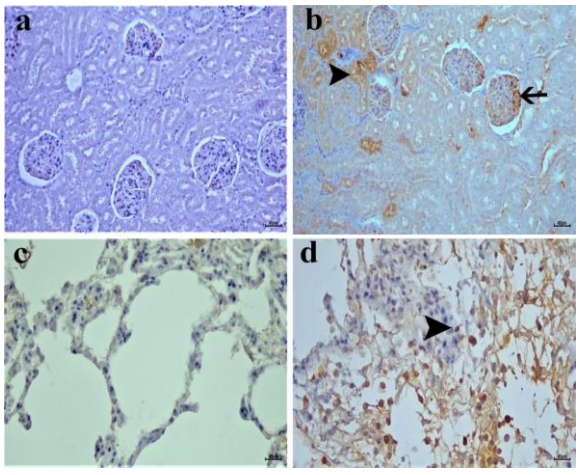

Figure 5. a) Sham group; kidney tissue negativity for caspase-3 immunopositivity. b) CLP group; intense Caspase-3 immunopositivity in tubular epithelium (arrowhead) and glomerulus (arrow), c) Sham group; lung tissue negativity for caspase-3 immunopositivity, d) CLP group; intense Caspase-3 immunopositivity in inflammatory cells in the alveolar space (arrowhead), IHCx20.

DISCUSSION

Sepsis is a worldwide health problem enhancing death rates in intensive care units (Benz et al., 2016). It may result from various conditions, including trauma, surgery, and infection (Lan et al., 2017). An intestinal leakage-related microbial inflow to the peritoneal cavity may lead to sepsis (Angus & Wax, 2001). CLP is admitted a pragmatic experimental model to create realistic polymicrobial sepsis and it has been used and improved for over 30 years (Remick et al., 2000; Wichterman et al., 1980). CLP demonstrates similar symptoms to sepsis (D. Rittirsch et al., 2009).

During sepsis, an excessive inflammatory response occurs causing multiple organ failures (Prescott & Angus, 2018). Lungs (Andrews et al., 2006) and kidneys (Lan et al., 2017) are among the major affected organs by sepsis. During sepsis, oxidative stress harms the lungs and various proinflammatory cytokine levels increase including IL-1β and IL-6 (Akpinar et al., 2014). A different study demonstrated high IL-1β levels due to CLP-induced sepsis in rats (Liu et al., 2016). IL-1β and IL-6 levels were raised in another CLP rat model (Zhao et al., 2018). IL-10 production is a strong response to an excessive inflammatory process (Oberholzer et al., 2002). Previous research has established that a CLP-induced sepsis model elevated IL-1β, IL-6, and IL-10 levels (Ruiz et al., 2016). It has been suggested that IL-1β, IL-6, and IL-10 increased in a rat model of CLP (Hu et al., 2019). In the current study, we observed high IL-1β, IL-6 levels indicating an inflammatory process, and we also obtained high IL-10 levels compatible with an answer against inflammation due to sepsis.

GSH, a component of the antioxidant system, defends the body against oxidative stress (Rahman et

al., 2004). GSH was observed as decreased in a CLP rat model (Tripathi et al., 2022). SOD activity represents the removal of superoxide anions as a part of the antioxidant system and MDA may reflect the ROS production (Fang et al., 2002). ROS production promotes neutrophil infiltration (Liu et al., 2019) during the sepsis-related inflammatory response. MPO settles mainly in neutrophils and may reflect the neutrophil infiltration (Wang et al., 2019).

Akpınar et al. showed that SOD and GSH values declined, and MDA levels raised in a CLP-induced rat sepsis model (Akpinar et al., 2014). In another CLP-induced sepsis model, sepsis diminished SOD and GSH levels, and increased MPO values (Cadirci et al., 2010). MPO activity and MDA value increased, but GSH and SOD levels raised in a previous CLP-induced rat sepsis model (Liu et al., 2016). In our study, we obtained low SOD and GSH values, but high MDA and MPx levels which are compatible with these results.

Sepsis causes damage due to excessive inflammatory responses of the body. The damage may be shown as histopathological changes in the tissues (Liu et al., 2016; L. Song et al., 2018). We observed changes in renal and lung tissues on behalf of sepsis compatible with previous studies. Caspase-3 involves in apoptotic pathways (Yu Wang et al., 2018). Many recent studies have shown that Caspase-3 expression raises during sepsis (Kostakoglu et al., 2020; Yu Wang et al., 2018; Zhao et al., 2018). We found Caspase-3 immunopositivity in our tissue samples indicating sepsis-related damage which is similar to the literature. Histopathological and immunohistochemical data supported the efficiency of the current sepsis model in addition to the biochemical findings.

CONCLUSIONS

The current study has presented a mid-grade CLP-induced rat sepsis model. We assessed blood cytokine levels and tissue oxidant/antioxidant parameters. In addition, we examined renal and lung tissues for histopathological and immunohistochemical evaluation. The results demonstrated a meaningful sepsis picture indicating a successful CLP model in concert with the literature which we may prefer to use in further CLP-induced sepsis models.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This study was designed to create and improve a CLP animal model for further CLP-related studies. It was supported by Atatürk University Department of Scientific Research Project as a research start-up support project (Project ID:8791).

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