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The effects of hydroxyurea on proinflammatory cytokine and tissue histopathology in an experimental sepsis model

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Abstract. – OBJECTIVE: The diagnosis and treatment of sepsis are costly to healthcare services, and it is an important disease with high mortality rates. In the pathogenesis of sepsis, for which we still cannot provide a complete cure, there is increased cytokine release and organ damage. Hydroxyurea has been shown to reduce leukocyte counts, decrease inflammatory cytokines, and limit organ inflammation in ischemia-reperfusion models. This study aimed to evaluate leukocyte counts, interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) cytokine values and organ inflammatory processes in hydroxyurea-treated rats with an experimental sepsis model.

MATERIALS AND METHODS: After ethical approval, rats were randomly divided into three groups, control (n= 7), sepsis (n= 7), and hydroxyurea (n= 7). Sepsis was created using the cecal ligation and puncture (CLP) method in rats other than in the control group. Rats in the hydroxyurea group received hydroxyurea (200 mg/kg) intragastrically, and the control and sepsis groups received sterile distilled water. IL-1β, IL-6, and TNF-α levels were measured at 0, 8, and 24 hours after CLP in all rats. Blood samples were collected at the time of sacrification 24 hours after CLP and analyzed for the complete blood count. Tissue specimens were taken for histopathologic examination.

RESULTS: Cytokine levels (IL-1β, IL-6, TNF-α), white blood cell counts, and tissue damage were increased after the sepsis model in rats. It was found that the cytokine levels at the 8th hour, white blood cell count, and brain tissue damage in the hydroxyurea group were decreased significantly compared with the sepsis group.

CONCLUSIONS: Early hydroxyurea treatment in rats with sepsis decreases proinflammatory cytokine (IL-1β, IL-6, and TNF-α) levels and thus reduces brain damage.

Key Words:
Cytokines, Sepsis, Brain.

Introduction

Sepsis, according to the latest definition "Sepsis 3" in 2016, is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection and is a leading cause of mortality and morbidity worldwide[1]. Although exact numbers are unavailable, some 2.8 million global deaths every year are attributed to sepsis[2]. The pathogenesis of the disease involves the hyperinflammatory host response to the pathogen and the immunosuppressive state following this proinflammatory state[3]. The precise mechanisms of cell injury and sepsis-induced organ dysfunction are not fully understood and continue to be an active area of scientific investigation[2].

Hydroxyurea, which was first identified by Dresler and Stein in 1869 in Germany, blocks DNA synthesis by inhibiting ribonucleotide reductase, and mainly affects the cells that actively synthesize DNA[4,5]. This effect is reversible, with the inactivated enzyme being spontaneously regenerated upon the removal of hydroxyurea[4], which was approved by the United States Food

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and Drug Administration (FDA) for use as a cancer drug in 1967, and it is currently used to treat sickle cell anemia, HIV, psoriasis, and myeloproliferative diseases[5-7].

Several studies have shown hydroxyurea to reduce pro-inflammatory cytokines and inhibit the inflammatory process in tissues after ischemia-reperfusion models[8,9]. A review of the literature revealed no study of the effect of hydroxyurea, if any, on the proinflammatory process in sepsis, and its protective effects on associated organ dysfunction. Accordingly, the present study investigates the potential effect of hydroxyurea on proinflammatory cytokine and tissue histopathology in experimental sepsis.

Materials and Methods

Ethical Statement

The animal experiments were approved by the Gazi University Animal Experiments Local Ethics Committee (Ankara, Turkey, Approval No: G.Ü.ET-18.004, January 16th, 2018). The experiments were conducted at the Gazi University Laboratory Animal Breeding and Experimental Research Center between July 23rd, 2018, and July 26th, 2018.

Animals

A total of 21 male Sprague Dawley rats (aged 10-12 weeks) weighing 250-300 g were involved in the study, which were obtained from Gazi University Laboratory Animal Breeding and Experimental Research Center. The rats were maintained in a 12-h light/ dark cycle at 25°C, with a maximum of four rats housed per cage. The rats were provided standard rat chow and water ad libitum throughout the study. All rats were cared for and used according to the principles of the Guide for the Care and Use of Laboratory Animals, formulated by the Institute of Laboratory Animal Resources and produced by the National Institutes of Health (www.aaalac.org; 8th edition; Washington DC, National Academic Press, 2011).

Experimental Design

Study Groups

The rats were divided randomly into three groups: (i) control group (group C, n= 7), (ii) sepsis group (group S, n= 7), and (iii) the hydroxyurea group (group H, n= 7). The rats in group C underwent sham surgery, which involved a midline laparotomy and the exteriorization of the cecum only. The rats in group S and group H underwent CLP operations. Hydroxyurea (Hydrea® 500 mg capsule; Corden Pharma Latina, Italy) was diluted in sterile distilled water (100 mg/mL) immediately before use. Hydroxyurea (200 mg/kg) was administered to the rats in group H, and an equal volume of sterile distilled water was administered to the rats in groups C and S by oral gavage before the operation. The hydroxyurea dose used in the research was selected based on the findings of previous experiments[9-11].

Sepsis Model

At the beginning of the experimental procedure, anesthesia was induced in all rats using an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar®; Pfizer, Istanbul, Turkey) and 5 mg/kg xylazine (Rompun®; Bayer, Turkey). After shaving the abdominal hair and cleaning the area with povidone-iodine, a 2-cm midline abdominal incision was performed, and the cecum was exposed. CLP was performed as previously described[12]. Briefly, the cecum was ligated just below the ileocecal valve and punctured twice using an 18-gauge needle, and then replaced into the abdominal cavity. The incision was then closed in layers. For the sham surgery, the cecum was exposed as previously described, but was neither ligated nor punctured. All rats were resuscitated using normal saline (30 mL/kg per body weight) subcutaneously after surgery and returned to their cages.

Data Collection

Blood samples were collected from the tail veins of the rats after the surgical procedure was completed, and further blood samples were taken at 8 and 24 hours after CLP. The whole blood was centrifuged at 3000 g at 4°C for 15 minutes, after which the plasma was separated and stored at -80°C until the laboratory analysis of cytokines. At the end of the experiment, intracardiac blood was collected under deep anesthesia and the rats were sacrificed 24 hours after CLP. Brain, heart, lung, liver, and kidney tissue samples were collected for histologic analysis. All samples were numbered.

Laboratory Analysis of Blood Samples

A complete blood count was made using the automated impedance analysis method (Beck-

man Coulter® LH 780, Brea, CA, USA) in the University of Health Sciences Diskapi Yildirim Beyazit Training and Research Hospital Clinic Biochemistry Laboratory (Ankara, Turkey) by the researcher, who was blinded to the group allocation.

Measurement of Plasma Cytokines

Systemic cytokines, including IL-1β, IL-6 and TNF-α were measured using enzyme-linked immunosorbent assay kits (USCN Life Science, USA), following the manufacturer's instructions.

Histologic Analysis

Tissues were collected from the brain, heart, kidney, liver and lung 24 hours after CLP, and each sample was fixed in 10% neutral buffered formalin. Histologic examinations were performed in the University of Health Sciences Diskapi Yildirim Beyazit Training and Research Hospital Pathology Laboratory by a pathologist who was blinded to the treatment. The samples were embedded in paraffin, and sections were cut from the paraffin blocks and stained with hematoxylin-eosin. Histologic changes were evaluated using a standard light microscope (Olympus BX 53 microscope, Tokyo, Japan) and photographed. The evaluations were made considering the histopathologic findings of tissue damage and inflammation specific to each organ. Lung tissue sections were evaluated using a scoring system as previously reported[13]. Briefly, lung injury was scored on a scale of 0 (normal) to 4 (severe), including congestion, hemorrhage, leukocyte infiltration and neutrophil aggregation. Histopathologic liver injury was evaluated as previously described and graded from 0 (normal) to 4 (severe), as follows: hemorrhage, hepatic parenchymal and sinusoidal inflammation, and hepatocellular necrosis[14]. The kidney sections were evaluated for tubular atrophy, dilatation, widening of the interstitium, and cellular infiltration. The degree of renal damage was determined using a graded scale as previously reported, where 0=normal, 1=minimal (damage of <25%), 2=mild (damage of 25-50%), 3=moderate (damage of 50-75%), and 4=severe (damage of >75%)[15].

Histopathologic analysis of heart injury was made as previously described[16]. Briefly, heart injury was graded from 0 (normal) to 2 (severe) as follows: infiltration and degeneration of muscle. The brain tissue sections were evaluated as previously described and scored on a scale of 0 (normal) to 2 (severe) in the following categories: degeneration of neurons, vascular edema, and hemorrhage[16].

Statistical Analysis

The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 11.5 software package (SPSS Inc., Chicago, IL, USA). Quantitative variables are presented as a mean± standard deviation (SD) and qualitative variables are expressed as frequencies (percentage). The group data were compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. Chi-square and Fisher's exact tests were used to establish the relationship between two qualitative variables. A paired *t*-test was used to determine the difference between two dependent quantitative variables. The significance level was set at a *p*-value of < 0.05. Analyses were performed by a professional statistician who was blinded to the group allocations.

Results

Complete Blood Count Analysis

All 21 rats survived the 24 h observation period. The white blood cell (WBC) count was statistically significantly higher in the sepsis group (2.58 ± 0.99 $10^3/\mu L$) than in the control group (0.94 ± 0.42 $10^3/\mu L$, $p=0.004$). In group H, which received hydroxyurea treatment, this value (1.50 ± 0.40 $10^3/\mu L$) was significantly lower than in the sepsis group ($p=0.04$). No significant difference was found between the other results of complete blood counts for the three groups.

Plasma Cytokines

The plasma concentrations of IL-1β, IL-6, and TNF-α were very low in the control group and increased in the rats undergoing CLP at both 8 h and 24 h (Table I). The increase in all cytokine concentrations 8 h after CLP was significantly attenuated by hydroxyurea treatment ($p<0.05$; Figure 1). No significant difference was established in the IL-1β or TNF-α levels of the sepsis and hydroxyurea groups 24 h after the experiment (Figure 1). However, the hydroxyurea treatment was found to inhibit the increase in IL-6 concentration at 24 hours ($p= 0.047$, Figure 1).

Table I. Plasma cytokine results of groups.

Hour	Group C Mean ± SD	Group S Mean ± SD	Group H Mean ± SD	*p*-value
IL-1β (pg/ml)				
0	33.93 ± 8.24	39.20 ± 8.12	37.19 ± 6.49	0.310[b]
8	35.95 ± 7.85	140.84 ± 33.55	88.09 ± 19.97	< 0.001[a]
24	34.85 ± 7.42	72.16 ± 22.32	83.84 ± 24.00	< 0.001[a]
IL-6 (pg/ml)				
0	38.34 ± 20.23	42.44 ± 14.87	39.74 ± 8.96	0.880[a]
8	42.21 ± 18.65	4134.44 ± 877.39	1807.55 ± 428.63	< 0.001[a]
24	43.94 ± 15.82	839.37 ± 148.01	742.51 ± 120.13	< 0.001[a]
TNF-α (pg/ml)				
0	58.39 ±15.72	69.22 ± 17.46	64.31 ± 19.23	0.524[a]
8	60.61 ± 16.08	369.51 ± 50.50	220.17 ± 61.47	< 0.001[a]
24	57.35 ± 15.02	171.40 ± 34.88	179.83 ± 29.09	< 0.001[a]

[a]: One Way ANOVA test, [b]:Kruskal Wallis H test.

Histologic Analysis

The histologic findings included normal cell structures in the brain, heart, lung, liver, and kidney of the control group. Histopathologic changes such as inflammatory cell infiltration, congestion, necrosis, and degeneration were observed in tissue sections of the rats undergoing CLP. There were no significant differences in the histopathologic findings related to the heart, lung, liver, and kidney of the group S and group H rats. Hydroxyurea treatment only inhibited pathologic changes in brain tissue (p=0.04, Figure 2).

Discussion

Despite significant developments and advancements in sepsis therapy, the life-threatening complications of sepsis have led to hundreds of thousands of deaths in the United States and millions more worldwide[17,18]. In the present study, a new treatment approach was administered in a CLP-induced sepsis model. Pretreatment with hydroxyurea was found to significantly inhibit the elevation of serum IL-1β, IL-6, and TNF-α concentrations in the early period, and to reduce brain injury caused by sepsis.

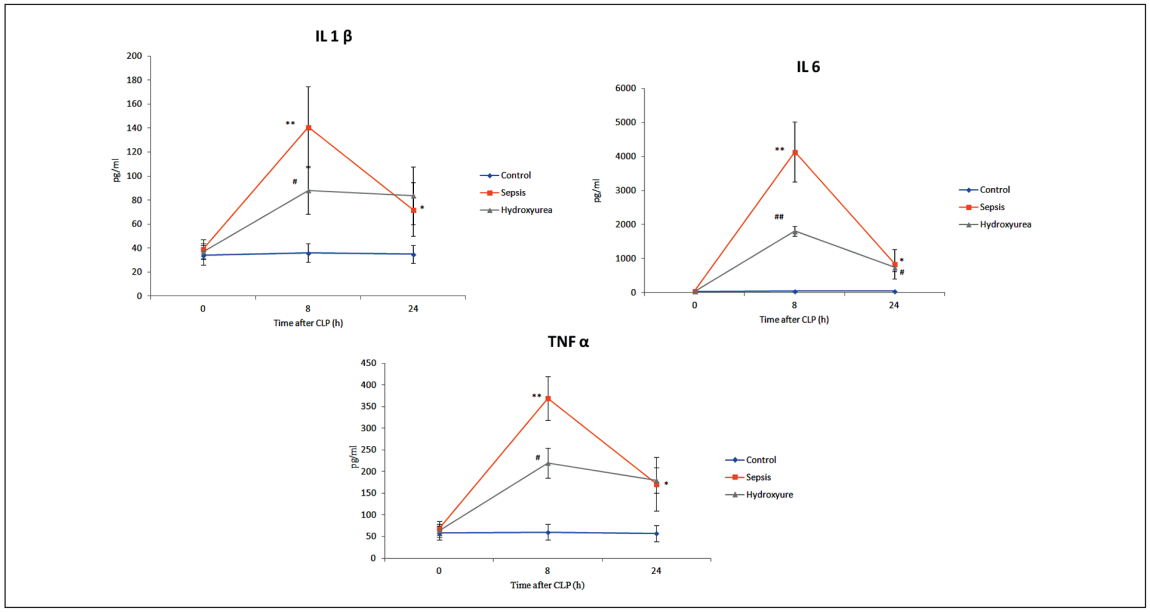

Figure 1. The plasma concentrations of IL-1β **(A)**, IL-6 **(B)**, and TNF-α **(C)** in rats of the control, sepsis, and hydroxyurea groups. The results are presented as mean±standard deviation. * or ** compared with the control group p<0.05 or p<0.01; # or ## compared with the sepsis group p<0.05 or p<0.01.

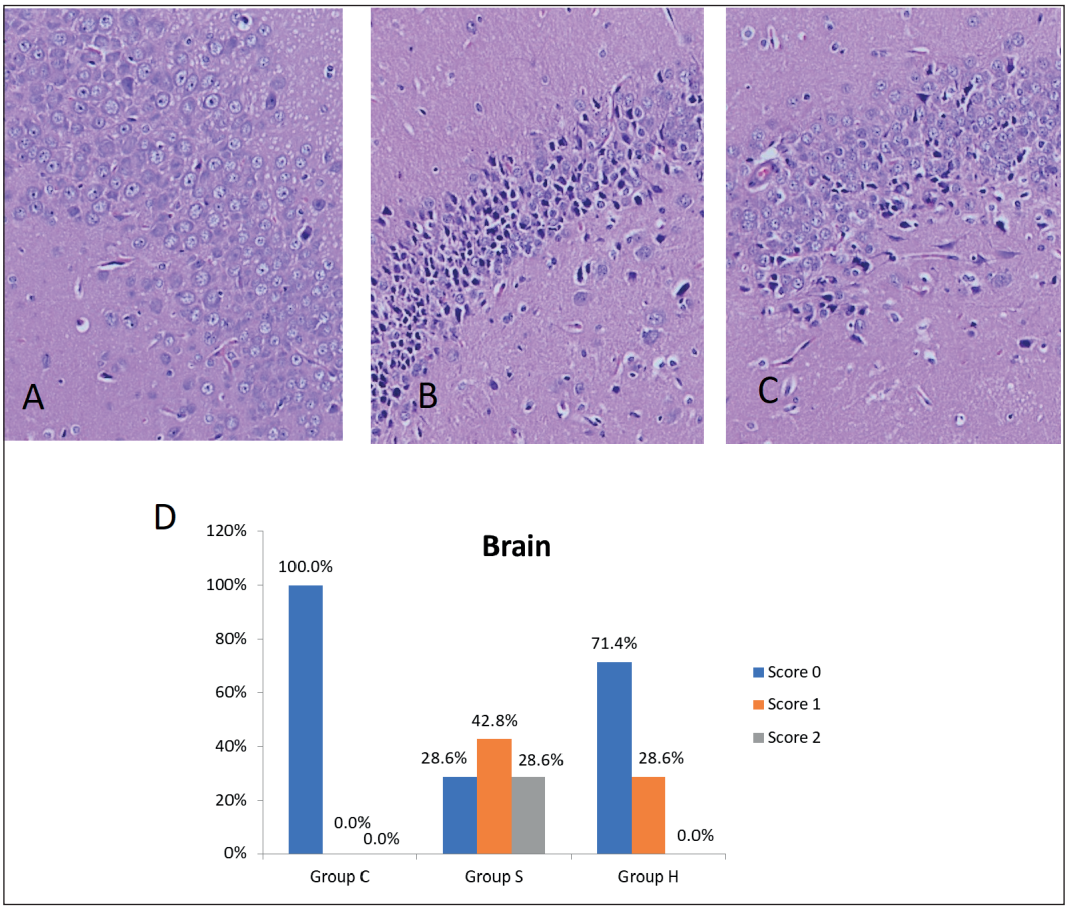

Figure 2. Histologic assessment of rat brains. Representative images, hematoxylin-eosin stained brain sections, were chosen from the control group **(A)**, sepsis group **(B)**, and the hydroxyurea group **(C)** (magnification ×200). Semiquantitative analysis of brain injury **(D)**. Group C: control group (n= 7), group S: sepsis group (n= 7), and group H: hydroxyurea group (n= 7). Score 0: normal, 1: mild, 2: severe injury.

The CLP procedure, which is considered an appropriate model for the induction of polymicrobial sepsis in an experimental setting and the examination of the mechanisms underlying sepsis, has been demonstrated to provide a clear understanding of sepsis-induced hypermetabolic and hypometabolic processes in humans[12,19,20]. While inducing a sepsis model, punctures can be made in different numbers using different needle sizes[12,19-22]. It has been reported that the material used for the perforation and the size of the perforation in CLP has different effects on sepsis induction, with a puncture with an 18-gauge venous catheter producing TNF-α levels equivalent to the intraperitoneal administration of 80 mg/kg endotoxin after 5 hours[23]. In the present study, punctures were made twice using an 18-gauge needle, the effectiveness of which is well established[23,24].

Hydroxyurea reaches the peak plasma blood level within 1-4 hours of intestinal absorption after oral intake. In 1975, Timson[25], a geneticist, found that hydroxyurea exerted its effects on cellular mitosis and karyorrhexis within 1 to 4 hours of maximum plasma concentration, and such effects were restored to a level similar to that of normal cells after approximately 6 hours. Based on these data, the present study analyzed the plasma levels of cytokines in the first 8 hours after the experiment. In a study explaining organ dysfunction mechanisms in sepsis, Cinel et al[26] reported that inflammatory cytokines were observed at high levels within the first 24 hours. Taking this into account, the experiment in the present study was terminated at 24 h, and blood and tissue samples were examined.

In sepsis, first, an excessive inflammatory process is initiated, and the WBC count is increased[26,27]. In the present study, the WBC count was increased in the sepsis group compared with the control group. The WBC count,

in turn, was significantly lower in the hydroxyurea-treated rats than in the sepsis group ($p<0.05$). Hydroxyurea is known to produce this effect through its tendency to accumulate in rapidly proliferating cells such as bone marrow, erythrocytes, leukocytes, and malignant cells, and by inducing an S phase-specific arrest in cell division[28,29]. In the present study, the hydroxyurea treatment dose of 200 mg/kg inhibited leukocytosis but did not lead to complete immunosuppression during the inhibition. Zhu et al[9] conducted a myocardial infarction study on rats and reported a significant reduction in leukocytosis upon hydroxyurea treatment at a dose of 200 mg/kg, indicating that a decreased leukocyte load is associated with reduced mortality and morbidity. A toxicology study for hydroxyurea demonstrated that the gavage administration of 500 mg/kg caused major bone marrow depression and pancytopenia in rats[11]. The median lethal dose of hydroxyurea in rats is 5780 mg/kg in a single oral dose[30]. In light of these data, it is believed that the hydroxyurea dose of 200 mg/kg used in the present study was within the effective and safe range.

During the first process, in which inflammatory cytokines are predominant, the primary actors are TNF-α, IL-1β, and IL-6, for which the main production source is leukocytes. This was proven by Wang et al[32] through the RNA expression of the related cytokines. Several studies have identified a strong correlation between decreased plasma concentrations of these cytokines and mortality[32-35]. In the present study, these cytokines were not different between the groups at the start of the experiment, whereas hydroxyurea treatment inhibited the excessive increase of these cytokines in sepsis at 8 h (Figure 1). Experimental models in the literature have made use of several methods to inhibit cytokine elevation in inflammatory states using molecules such as ketamine, bupivacaine, lidocaine, and propofol[21,35-37]. However, all of these practices, despite being steps for ongoing studies, are still absent from the current sepsis guidelines[31]. Hydroxyurea, based on the findings of the present study, appears to be a new molecule open to research in many ways.

At 24 h following the experiment, cytokine concentrations were similar in the treated and untreated septic rats, which were attributed to the short half-life of hydroxyurea, its reversible effects, and the dose of the drug. The half-life of hydroxyurea is 1-5 hours after oral intake, with 80% excreted by the kidneys within 12 hours[28]. It is considered acceptable that this drug, which has a rapid distribution, action, and metabolism, was not observed to have any lasting effects 24 h after oral intake by gavage in a single dose.

The present study established the most significant difference between the groups was in terms of organ injury, specifically, in brain tissue. The inflammatory changes in other tissues were mild but moderate and severe in the brain tissues of the sepsis group. These inflammatory changes were observed to be significantly lower in animals undergoing hydroxyurea treatment (Figure 2). Although the heart, lungs, and kidneys are the main organs in which sepsis-induced multiple organ dysfunction develops, the brain injury that occurs in the early phase is a remarkable consequence of sepsis. Brain injuries occur in the early phase of sepsis and contribute to its progression[38,39].

The relatively early sacrification and the lack of a long-term assessment of organ dysfunction may be considered limitations of the present study. A larger number of subjects would help to achieve more statistically significant results; however, the number of rats was kept at a minimum due to ethical concerns. Further studies into the optimal dose and time of administration will produce valuable data in terms of the clinical applicability of hydroxyurea in sepsis. An assessment of the results following the above-mentioned method will provide additional support to *in vivo* human studies.

Conclusions

Sepsis remains one of the leading causes of mortality in intensive care units. Despite technological advancements, there is a need for effective and focused treatment methods. The present study demonstrated that early treatment with hydroxyurea-controlled leukocyte counts and early cytokine levels in an intra-abdominal sepsis model induced using CLP. We believe that the inflammatory effects of sepsis on brain tissue may have been reduced through the decreased levels of cytokines and suggest that clear evidence related to other tissues may be achieved with a larger number of rats and a longer period before sacrification.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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