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EXPERIMENTAL STUDY

Comparison of effects of quercetin and ascorbic acid on inflammatory cytokines and antioxidant biomarkers in infant rats using an experimental sepsis model

Emine Ufuk BOZKURT[1], Abdulrahman OZEL[1], Meltem EROL[1], Aslihan TENEKECIGIL[2,3], Ozlem BOSTAN GAYRET[1], Ovgü BUKE[1], Volkan TOSUN[1]

Health Sciences University Turkey, Bağcilar Training and Research Hospital, Clinic of Pediatrics Istanbul, Turkey.
dr.abdulrahman.ozel@gmail.com

ABSTRACT
OBJECTIVE: There is ongoing research on treatments that promote antioxidant and anti-inflammatory mechanisms, which will reduce mortality in sepsis. In this study, we compared the anti-inflammatory and antioxidant activities of quercetin and ascorbic acid using a sepsis model induced in infant rats.
METHODS: A total of 28 infant rats 21-days-old that had just completed the lactation period were divided into four groups: control, sepsis, sepsis + quercetin, and sepsis + ascorbic acid. The sepsis model was created with an intraperitoneal injection of bacterial lipopolysaccharide. After 24 hours, blood samples were collected for analysis of serum levels of inflammatory cytokines (IL-1β, IL-6, TNF-α, and CRP) and antioxidants (CAT, GPx, SOD, and GST).
RESULTS: The superoxide dismutase levels were significantly higher in the sepsis + ascorbic acid group compared to the sepsis and sepsis + quercetin groups. The levels of the most active cytokines in sepsis were significantly lower in the serum samples of the septic subjects who received quercetin and ascorbic acid.
CONCLUSION: The antioxidant activity, which is impaired in sepsis, was increased by both molecules. We observed that these two molecules, which are free of side effects, have a positive influence on the progression of sepsis to severe and fatal sepsis in childhood *(Tab. 2, Ref. 38)*. Text in PDF *www.elis.sk*
KEY WORDS: antioxidants, ascorbic acid, quercetin, sepsis, cytokines, infant rats.

Introduction and objective

Sepsis is a life-threatening organ failure caused by an irregular host response to infection. It is a complex clinical condition characterized by oxidative stress and inflammation induced by the host in response to the damage caused by microorganisms (1). It is a serious public health problem that affects millions of people worldwide and is fatal in one in 3 to 6 cases (2).

The liposaccharides (LPS) found in the cell wall of the Gram-negative bacteria are an important pathogenic factor in sepsis due to their endotoxin properties. For years, several studies have been conducted in experimental animal models to elucidate this issue. One of the most commonly used methods is LPS injection. Systemic inflammation induced by LPS mimics the clinical process of sepsis (3).

Although there have been several developments in this field and an increasing number of studies in the diagnosis and treatment of sepsis and septic shock in recent years, mortality from sepsis remains very high (2). The search is on for treatment methods that are effective on the antioxidant and anti-inflammatory pathways that will reduce mortality in sepsis (4). Ascorbic acid is well known to have a positive effect on the immune system and antioxidant activity by reducing the production of inflammatory cytokines (5). However, there are only a very limited number of studies in the literature focusing on the anti-inflammatory and antioxidant effects of quercetin – a herbal flavonoid – on sepsis. Flavonoids are a group of naturally occurring substances that have various biological properties including anti-inflammatory and antioxidant activity (6, 7). Quercetin is found in many vegetables, fruits, and drinks and has several physiological properties including anti-inflammatory, antiproliferative, and antioxidant activity. Therefore, it is an important component of human nutrition. Recent studies in the literature have shown that quercetin may decrease the release of TNF-α and IL-1β and consequently alleviate inflammatory responses (8). Although the anti-inflammatory activity of quercetin is well known, its role in preventing mortality and systemic inflammation in patients with fatal sepsis remains to be elucidated.

[1]Health Sciences University Turkey, Bağcilar Training and Research Hospital, Clinic of Pediatrics Istanbul, Turkey, [2]Department of Medical Biochemistry, Health Science University, Bagcilar Research and Education Hospital, Istanbul, Turkey, and [3]Department of Medical Biochemistry, Faculty of Medicine, Gazi University, Ankara, Turkey

Address for correspondence: Abdulrahman OZEL, MD, Health Sciences University Turkey, Bağcilar Training and Research Hospital, Clinic of Pediatrics Istanbul, Turkey. Phone: +905312058253

In this study, our objective was to evaluate the anti-inflammatory and antioxidant properties of quercetin and ascorbic acid separately by using a sepsis model induced in infant rats and to compare the effects of these two potent antioxidant molecules. Our findings may contribute to the literature by demonstrating that these antioxidant molecules can mitigate the severe clinical course of sepsis, which is a major cause of mortality, especially in children.

Materials and methods

In this study, we used 28 Wistar Hannover rats, which were obtained from the Experimental Research and Training Center Laboratory of Istanbul Bagcilar Training and Research Hospital at Health Sciences University. Twenty-one of the rats were male and 7 were female. They were 21 days old and weighed between 50 and 55 grams. The rats were fed with a standard diet and water and each group was kept in a special cage. The rats were exposed to a photoperiod of 12 hours of light and 12 hours of dark at a room temperature between 18 and 22 °C. They were divided into 7 rats per cage. The study was carried out in the Experimental Research and Training Center Lab of the Experimental Research and Training Center Lab of the Istanbul Bagcilar Training and Research Hospital at Health Sciences University and the study protocol was approved by the Local Ethics Committee for Animal Experiments in the our hospital (118th Committee meeting; Approval No: 2021-122). This animal study was conducted according to the standards recommended by the Council of Europe (*European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*) (*ETS 123*).

Experimental applications and study plan

A total of 28 infant rats 21-days-old that had just completed their lactation period, were divided into 4 groups. 1st group: Control group. The rats in the control group did not receive any drug supplement for 7 days. Only 1 ml of saline was administered by oral gavage. Sepsis model was not induced in these rats on the 8th day. 2nd group: Sepsis group. The rats in the sepsis group did not receive any drug supplement for 7 days, but 1 ml of saline was administered by oral gavage. 3rd group: Sepsis + quercetin group. Quercetin (25 mg/kg/day) was administered by oral gavage for 7 days. 4th group: Sepsis + ascorbic acid group. Ascorbic acid (200 mg/day) was administered by oral gavage for 7 days.

On the 8th day of the experiment, a sepsis model was created with intraperitoneal injection of bacterial LPS (30 mg/kg) in the rats of the 2nd, 3rd, and 4th groups.

In all groups, a 5 cc intracardiac blood sample was collected from each rat on the 9th day to assess the inflammatory cytokines levels (IL-1β, IL-6, TNF-α, and CRP) and antioxidant levels (catalase, glutathione peroxidase, superoxide dismutase, and glutathione S-transferase). Blood samples were centrifuged and stored at -80 °C until the analysis.

Measurement of inflammatory cytokines levels

TNF-α, IL-1β, and IL-6 levels were measured according to the procedures of the enzyme-linked immunosorbent assay (ELISA) method by using Farmasina Inc. kits. CRP measurements were carried out with the spectrophotometric method in the auto-analyzer using kits from Farmasina Inc.

Measurement of antioxidant levels

The superoxide dismutase (SOD) enzyme activity was measured with the Marklund and Marklund method (9). The method defined by Aebi was used for the determination of the catalase (CAT) enzyme activity (10). The method of Paglia and Valentine was preferred for the evaluation of the glutathione peroxidase (GPx) enzyme activity (11). The method defined by Habig et al was used for the evaluation of the glutathione S-transferase (GST) enzyme activity (12).

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics 22 (IBM SPSS, Turkey) software package. The normal distribution of the parameters was evaluated with the Shapiro-Wilk test. In addition to the descriptive statistical methods (frequency), the Kruskal-Wallis test was used to compare the parameters that did not show a normal distribution when comparing quantitative data. Dunn's test was used to determine the group responsible for the difference between the groups. The accepted limit of significance level was $p < 0.05$.

Results

A statistically significant difference was determined ($p = 0.000$; $p < 0.05$) between the groups when considering the CRP levels. The paired comparisons performed to determine the difference showed that the CRP levels in the control group were significantly lower than those in the sepsis and sepsis + ascorbic acid groups ($p1 = 0.000$; $p2 = 0.002$; $p < 0.05$). The CRP levels were also significantly higher in the sepsis group compared to those in the sepsis+quercetin group ($p = 0.015$; $p < 0.05$). There was no statistically significant difference in CRP levels between the other groups (Tab. 1).

Tab. 1. Evaluation of inflammatory markers between the groups.

Group	Control	Sepsis	Sepsis + quercetin	Sepsis + ascorbic acid	Total	p
	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	
CRP (ng/mL)	46.61±20.89 (42.5)	133.48±11.87 (136.4)	95.14±16.47 (99.4)	117.01±20.01 (116.6)	98.06±37.21 (100.6)	0.000*
TNF-α (ng/L)	132.82±47.17 (120.4)	226.09±32.57 (212.1)	153.85±44.74 (160.9)	185.6±28.01 (198.5)	174.59±51.31 (175.4)	0.006*
IL-1β (pg/L)	1265.1±297.13 (1100.4)	2282.92±306.33 (2119.1)	1713.57±207.29 (1659.9)	1793.73±368.11 (1959.8)	1763.83±464.07 (1800)	0.000*
IL-6 (pg/mL)	64.32±6.44 (61.9)	99.84±42.7 (83.4)	71.85±7.02 (70.2)	77.6±11.3 (74.5)	78.4±25.21 (73.3)	0.002*

Kruskal-Wallis Test *$p < 0.05$

There was also a statistically significant difference in the TNF-α levels between the groups (p = 0.006; p<0.05). The paired comparisons performed to determine the difference showed that TNF-α levels in the sepsis group were significantly higher than those in the control and sepsis + quercetin groups (p1 = 0.001; p2 = 0.010; p < 0.05). There was no statistically significant difference in TNF-α levels between the other groups (Tab. 1).

Statistically significant differences were found between the groups for IL-1β levels (p = 0.000; p < 0.05). The paired comparisons performed to determine the difference showed that IL-1β levels were significantly lower in the control group compared to the sepsis and sepsis + ascorbic acid groups (p1 = 0.000; p2 = 0.032; p < 0.05). In addition, the IL-1β levels were significantly higher in the sepsis group compared to the sepsis + quercetin and sepsis + ascorbic acid groups (p1 = 0.008; p2 = 0.035; p < 0.05). There was no statistically significant difference in IL-1β levels between the other groups (Tab. 1).

A statistically significant difference was also found between groups for IL-6 levels (p = 0.002; p < 0.05). The paired comparison analysis performed to determine the difference showed that IL-6 levels were significantly lower in the control group than the IL-6 levels in the sepsis and sepsis + ascorbic acid groups (p1 = 0.000; p2 = 0.020; p < 0.05). Besides, the IL-6 levels were significantly higher in the sepsis group than the IL-6 levels in the sepsis + quercetin group (p = 0.017; p < 0.05). There were no other statistically significant differences in the IL-6 levels between the other groups (Tab. 1).

There were statistically significant differences in CAT levels between the groups (p = 0.002; p < 0.05). The paired comparison test performed to detect the differences showed that CAT levels in the sepsis group were significantly lower than the CAT levels in the control, sepsis + quercetin, and sepsis + ascorbic acid groups (p1 = 0.000; p2 = 0.003; p3 = 0.015; p < 0.05). There were no statistically significant differences in CAT levels between the other groups (Tab. 2).

There was a statistically significant difference in SOD levels between the groups (p = 0.001; p < 0.05). The paired comparison tests displayed that the SOD levels were significantly higher in the control group compared to the sepsis and sepsis + quercetin groups (p1 = 0.019; p2 = 0.001; p < 0.05). In addition, the SOD levels in the sepsis + ascorbic acid group were significantly higher than the SOD levels in the sepsis and sepsis + quercetin groups (p1 = 0.029; p2 = 0.001; p < 0.05). There was no statistically significant difference in SOD levels between the other groups (Tab. 2).

We also determined statistically significant differences in the GPx levels between the groups (p = 0.007; p < 0.05). The paired comparison tests showed that the GPx levels were significantly lower in the sepsis group compared to the control and sepsis + ascorbic acid groups (p1 = 0.003; p2 = 0.007; p < 0.05). There was no statistically significant difference in GPx levels between the other groups (Tab. 2).

Discussion

In sepsis, early diagnosis and treatment are critical to prevent severe sepsis and progression to a septic shock. Several studies focusing on the physiopathology of sepsis demonstrated that bacterial exotoxins lead to the release of cytokines and activate mediator systems. Endotoxins released by Gram-negative bacteria stimulate the production of biomarkers such as TNF-α, IL-1β, IL-6, and CRP, which worsen the clinical course of sepsis by activating the coagulation and complement cascades (13). Free radicals are also released along with inflammation and trigger the synthesis of cytokines. Therefore, it has been suggested that antioxidant treatment might be beneficial in addition to antibiotics in the treatment of sepsis (14).

Intraperitoneal administration of *Escherichia coli* LPS to experimental animals causes inflammation (15). This method is widely used to induce sepsis in experimental animals (16).

It has been observed that sequential measurements of CRP are most useful in giving an idea about the course of neonatal sepsis. Based on these observations, it is recommended to measure the CRP levels at least twice with a maximum of 12–24-hours between measurements (17). In a study conducted by Alpha A Fowler et al in 24 patients, ascorbic acid significantly decreased the CRP and PCT levels (18). In a study on sepsis and ascorbic acid conducted by Equey et al in Switzerland in 60 patients aged 0 to 16 years, the investigators showed higher CRP levels in patients receiving low-dose vitamin C supplements (19). Regarding the effects of quercetin on CRP levels, the study conducted by Garcia et al showed that quercetin administration could reduce CRP levels. In this preclinical *in vitro* study, the levels of inflammatory cytokines like NO-synthetase, COX-2, and CRP were decreased in the human hepatocyte-derived cell line (20). In another rat study conducted by Guardia et al, the investigators observed that quercetin inhibited both acute and chronic inflammation and significantly decreased the CRP levels compared to the control group (21). In our study, the CRP levels were significantly lower in the control group compared to the sepsis and sepsis + ascorbic acid groups (p < 0.05).

Tab. 2. Evaluation of antioxidant levels between the groups.

Group	Control	Sepsis	Sepsis + quercetin	Sepsis + ascorbic acid	Total	p
	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	Mean±SD (median)	
CAT (ng/mL)	49.15±7.28 (51.8))	28.32±6.12 (25.6))	46.22±7.91 (45.6))	43.69±6.05 (44.5))	41.85±10.45 (44.3))	0.002*
SOD (ng/mL)	22.63±3.79 (21.4))	17.32±3.13 (18.3))	14.69±2.63 (15.3))	21.55±2.78 (21.4))	19.05±4.39 (19))	0.001*
GPX (ng/mL)	35.2±8.95 (37.6))	21.23±5.69 (21.3))	26.27±6.29 (27.5))	33.8±5.87 (33.9))	29.13±8.66 (29.2))	0.007*
GST(ng/mL)	28.51±2.71 (27.8))	28.15±3.54 (27.7))	24.69±3.18 (25.2))	31.33±6.68 (30.7))	28.17±4.72 (27.5))	0.103

Kruskal-Wallis Test *p < 0.05

In addition, the CRP levels in the sepsis group were significantly higher than the CRP levels in the sepsis + quercetin group ($p < 0.05$). The comparison of two supplement groups (quercetin and ascorbic acid groups) did not show a statistically significant difference between these groups. In conclusion, although quercetin and ascorbic acid separately decreased the CRP levels, there was no significant difference between these two groups. The results considering CRP levels in our study were consistent with the results reported in the literature.

Excessive production of TNF-α, IL-1β, and IL-6 causes tissue and cell damage and leads to multi-organ failure and subsequently to fatal sepsis (22). Therefore, any drug and food supplement that inhibit cytokine expression is very important for the treatment of sepsis. In an experimental animal study, which was conducted by Yu-Cheng Chang et al in America, the investigators induced sepsis in rats with LPS injection and observed that quercetin significantly reduced the production of TNF-α, IL-1β, and IL-6 (23). In another study, Tang et al investigated quercetin. In this study, a single dose of quercetin was administered to rats, and it was observed that quercetin prevented severe sepsis caused by LPS endotoxin. However, it was also noted that the decrease in the levels of these cytokines was transient but single-dose quercetin was still effective at the acute stage of the disease (24). In another animal study conducted by JiaJia Liu et al, the investigators evaluated the effects of quercetin only on the IL-6 release and found that IL-6 levels were significantly lower in rats receiving quercetin supplements (25). The first findings related to the clinical use of vitamin C had been also obtained in animal models. Later, a few completed human studies contributed to the findings related to the beneficial effects of vitamin C in sepsis. In a study by Sawyer et al, which was published in 1986, the investigators continued to treat 16 ARDS patients with IV vitamin C (1000 mg/6 hours, IV) and the control group with the standard of care of that time. They determined a dramatic reduction in the mortality rate in the vitamin C group. The mortality rate was 37 % in the vitamin C group and 71 % in the control group ($p < 0.01$) (26). In a study conducted by Fowler et al in 2014, the investigators observed that vitamin C levels were very low in patients with severe sepsis and intravenous vitamin C administration significantly decreased the mortality rate (18). In a study by Seno et al investigating the effects of ascorbic acid on inflammatory cytokines, they found that IL-1β and TNF-α levels were significantly lower compared to the control group (27). A study conducted by Kim et al investigated the effects of ascorbic acid on inflammatory cytokines. In this study, infant rats, which were infected with the Influenza A virus, received vitamin C supplementation, but no significant decrease in the IL-1β and TNF-α levels was observed (28). In our study, there were statistically significant differences in TNF-α levels between the groups ($p < 0.05$). The TNF-α levels were significantly higher in the sepsis group compared to the control and sepsis + quercetin groups ($p < 0.05$). However, there was no statistically significant difference between the sepsis + quercetin and sepsis + ascorbic acid groups for TNF-α levels. We found that quercetin supplementation decreased the TNF-α levels. This finding was consistent with the findings in the literature. Regarding another parameter of our study, IL-1β levels were significantly lower in the control group compared to the sepsis and sepsis + ascorbic acid groups ($p < 0.05$). The IL-1β levels in the sepsis group were significantly higher compared to the sepsis + quercetin and sepsis + ascorbic acid groups ($p < 0.05$). The IL-6 levels were also significantly lower in the control group compared to the sepsis and sepsis + ascorbic acid groups ($p < 0.05$). Besides, the IL-6 levels in the sepsis group were significantly higher than the IL-6 levels the sepsis + quercetin group ($p < 0.05$). The comparison of quercetin and ascorbic acid groups for these two parameters did not show any statistically significant difference.

Depending on the reactive structure of free oxygen radicals, antioxidant systems are responsible for the prevention of tissue damage due to the oxidation of carbohydrates, proteins, and lipids (29). Enzymes such as SOD, CAT, GPx, and GST are endogenous enzymatic antioxidants and constitute the primary intracellular defense system (30). In general, elements of the antioxidant system remove the harmful oxygen derivatives (31). In the study conducted by Hsiao et al, it was reported that the SOD enzyme facilitates the conversion of reactive oxygen species to hydrogen peroxide and molecular oxygen in hepatocytes (32). In their study, Sebai et al showed that the activities of SOD, CAT, GPx, and GST were significantly low in the LPS administration groups (33). In another experimental animal study, Mohamadin et al induced sepsis with LPS injection and reported that CAT, SOD, and GPx enzyme levels were significantly lower in sepsis-induced groups (34).

To date, there is not much research on the effects of quercetin on antioxidant enzyme levels in sepsis. Gerin et al investigated the effects of single-dose quercetin in rats with induced sepsis in their study conducted in 2015. In this study, it was observed that quercetin was beneficial in acute liver injury by decreasing the levels of oxidative stress markers and increasing the antioxidant enzyme activities (35). In 2015, Huang et al induced sepsis in rats with LPS injection and found significant increases in the SOD, CAT, and GPx levels (36). Vitamin C has strong reductive properties. Vitamin C reacts with superoxide and hydroxyl radicals and plays an important role in their elimination from the body (37). Today, vitamin C is widely used depending on its potent antioxidant and anti-inflammatory properties. For years, its effects have been investigated in both children and adults. It has also been compared with other molecules, which were believed to possess antioxidant activity. In a study conducted by Noyan et al in 2004, vitamin A, E, and C supplementation and the antioxidant enzyme levels were compared in 50 infant rats. In this study, the investigators showed that vitamin C monotherapy may be more useful in reducing glucose levels and liver tissue lipid peroxidation compared to both insulin monotherapy and vitamin A and E treatment combined with insulin (38). However, there are only a limited number of studies focusing on the antioxidant effects of vitamin C in pediatric sepsis.

In our study, we determined that the CAT levels were significantly lower in the sepsis group compared to the control, sepsis+quercetin, and sepsis+ascorbic acid groups ($p < 0.05$). However, the comparison of sepsis+quercetin and sepsis+ascorbic

acid groups showed no statistically significant difference. On the other hand, SOD results were different from the CAT results. The SOD levels in the sepsis+ascorbic acid group were significantly higher compared to the sepsis and sepsis + quercetin groups ($p < 0.05$). We determined that vitamin C was more effective on SOD enzyme. The GPx levels in the sepsis group were significantly lower compared to the control and sepsis+ascorbic acid groups ($p < 0.05$). However, the comparison of GPx levels between the sepsis+quercetin and sepsis+ascorbic acid groups showed no statistically significant difference. There was also no statistically significant difference in GST levels between the groups ($p > 0.05$).

The most important limitation of our study was the need to work with a minimum number of animals due to ethical reasons and blood sampling procedure, which was carried out at a very short time interval. Comprehensive long-term studies with different dose levels will provide more elucidating information. The strength of our study is that no previous studies have focused on the comparison of the anti-inflammatory and antioxidant properties of quercetin and ascorbic acid in sepsis. Therefore, we believe that our study will make a significant contribution to the literature.

Conclusion

The physiopathology of sepsis is still under investigation. The importance of antioxidants is becoming apparent with the increasing number of studies, while the research on this topic is still ongoing.

There are only a limited number of studies in the literature focusing on the anti-inflammatory and antioxidant effects of vitamin C and herbal flavonoid quercetin on sepsis. In our study, we observed that these two molecules, which are free of side effects, have a positive influence on the progression of sepsis to severe and fatal sepsis in childhood.

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