

Effects of fullereneol C₆₀ on the liver, heart and brain tissues of streptozotocin-induced diabetic rats with sepsis

NECMIYE ŞENGEL¹, ÖMER KUBAT², LAMAN FARAJSOYLU³, AHMET ÇAĞRI BÜYÜKKASAP², MUHARREM ATLI⁴, ŞABAN CEM SEZEN⁵, HASAN BOSTANCI², KÜRŞAT DIKMEN², İŞİN GÜNEŞ⁶, FATMA ER⁷, MUSTAFA KAVUTÇU⁷ and MUSTAFA ARSLAN⁸⁻¹⁰

¹Department of Oral and Maxillofacial Surgery (Anesthesiology and Reanimation Specialist), Gazi University Faculty of Dentistry, Ankara 06490, Turkey; ²Department of General Surgery, Gazi University Faculty of Medicine, Ankara 06510, Turkey;

³Department of Dermatology, Lokman Hekim Hospital, Ankara 06010, Turkey; ⁴Department of Biology, Kırıkkale University Institute of Science and Technology, Kırıkkale 71450, Turkey; ⁵Department of Histology and Embryology, Kırıkkale University

Faculty of Medicine, Kırıkkale 71450, Turkey; ⁶Department of Anesthesiology and Reanimation, Erciyes University Faculty of Medicine, Kayseri 38030, Turkey; ⁷Department of Medical Biochemistry, Gazi University Faculty of Medicine,

Ankara 06510, Turkey; ⁸Department of Anesthesiology and Reanimation, Gazi University Faculty of Medicine, Ankara 06510, Turkey; ⁹Life Sciences Application and Research Center, Gazi University, Ankara 06830, Turkey;

¹⁰Laboratory of Animal Breeding and Experimental Research Center (GÜDAM), Gazi University, Ankara 06510, Turkey

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Abstract. In the present study, the protective effects of fullereneol C₆₀, a nanomaterial with antioxidant properties, were investigated against sepsis-induced multiple organ failure in liver, heart and brain tissues using a sepsis model [cecal ligation and puncture (CLP)] in diabetic rats. A total of 30 Wistar albino male rats were divided into four groups: Control, diabetic control, diabetes + sepsis (DM/SEP) and diabetes + sepsis + fullereneol C₆₀ (FUL/C-60). Streptozotocin was administered intraperitoneally at a dose of 55 mg/kg to induce diabetes in the rats; those with blood sugar levels >250 mg/dl 72 h after injection were considered diabetic. After 4 weeks, only laparotomy was performed in the control and diabetic control groups. CLP was applied to the DM/SEP and FUL/C-60 groups to establish a sepsis model. After laparotomy, the abdomens of the rats were closed. In the FUL/C-60 group, 100 mg/kg fullereneol C₆₀ was administered intraperitoneally 30 min after the procedure. All rats were sacrificed 24 h later and blood and tissue samples from the brain, liver and heart were taken for histopathological and biochemical examination. The data distribution was analyzed using the Shapiro-Wilk test. Data were evaluated using the Kruskal-Wallis test followed by Dunn's post hoc test or

one-way ANOVA followed by Tukey's post hoc test. Fullereneol C₆₀ markedly reduced sepsis-induced tissue damage in the liver, heart and brain. Histopathological evaluation demonstrated significantly less hepatocyte degeneration (P=0.042), necrosis (P=0.040) and sinusoidal dilatation (P=0.013) in liver tissue; reduced interstitial fibrosis (P=0.040) in heart tissue; and decreased inflammatory cellularity (P=0.034) in brain tissue in the FUL/C-60 group compared with the DM/SEP group. Biochemically, fullereneol C₆₀ significantly attenuated oxidative stress in liver and brain tissues, as indicated by lower thiobarbituric acid reactive substance levels (both, P<0.001) and higher catalase activity (P=0.034 and P=0.009, respectively) in the FUL/C-60 group. Consistent with these findings, liver function was substantially improved, as evidenced by reduced serum aspartate transaminase (P<0.001), alanine transaminase (P<0.001) and γ -glutamyl transferase (P=0.008) levels, as well as lower total (P<0.001) and direct bilirubin (P<0.001) levels. Overall, fullereneol C₆₀ effectively reduced organ damage by suppressing oxidative stress. Fullereneol C₆₀ demonstrated protective effects against multiple organ failure in a diabetic sepsis model, indicating its potential as a supportive agent in sepsis treatment.

Introduction

Use of nanomaterials in medicine began in the mid-20th century and has increased steadily in previous years (1,2). The ability to protect tissues from ischemia, as well as antimicrobial properties that may help defend the host against pathogens, has encouraged researchers to investigate carbon-based nanomaterials (2). Fullerenes have become a central focus of this research. The antioxidant properties of nanomaterials such as fullerenes, along with their protective effects against ischemia and antimicrobial activity, facilitate their potential

Correspondence to: Dr İşin Güneş, Department of Anesthesiology and Reanimation, Erciyes University Faculty of Medicine, Köşk Neighborhood, Dedefendi Street, Melikgazi, Kayseri 38030, Turkey
E-mail: isingunes@yahoo.com

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use as therapeutic agents (3-5). Among fullerene derivatives, fullereneol C₆₀ exhibits higher aqueous solubility than other fullerene structures due to the presence of hydroxyl groups on its surface (6). The water solubility of fullereneol C₆₀ makes it the most ideal nanomaterial among fullerenes, which also simplifies its use in *in vivo* (6,7).

The cecal ligation and puncture (CLP) method is a widely used procedure in animal models to induce polymicrobial sepsis and closely mimics the pathophysiology of clinical sepsis (8). This procedure involves surgically ligating a specific portion of the cecum and then puncturing it. CLP leads to an increased microbial load in the abdominal cavity and translocation of bacteria into the bloodstream, thereby triggering multiple organ failure, inflammation and immune responses that simulate clinical sepsis. Due to the immunological and physiological responses resembling clinical sepsis, this model is especially preferred in studies investigating sepsis pathogenesis and treatment (8,9). The CLP method is widely accepted as a reliable and reproducible experimental model for investigating sepsis-related complications and evaluating potential therapeutic interventions (8).

Sepsis is a life-threatening clinical condition caused by infection (10,11); it is estimated to account for ~49 million cases and ~11 million deaths worldwide each year, and remains one of the leading causes of mortality in Intensive Care Units (12).

Therefore, preventing sepsis or, if prevention is not possible, identifying effective treatment options is a primary focus for researchers. Although various therapeutic approaches, including early broad-spectrum antibiotics, fluid resuscitation, vasopressor support and adjunctive corticosteroid therapy, are routinely used in clinical practice, sepsis continues to be associated with high mortality, and no definitive therapy has yet demonstrated a consistent survival benefit (13). The development of multiple organ failure, particularly involving vital organs such as the liver, kidneys, lungs, brain and heart, presents significant challenges for clinicians, as it necessitates supportive interventions (for example, mechanical ventilation, vasopressor therapy and renal replacement therapy), complicates hemodynamic and metabolic stabilization, and is associated with increased morbidity and mortality (14). Sepsis is a significant global health problem; there are >30 million patients with sepsis worldwide every year (15). Sepsis-associated encephalopathy (SAE) is one of the symptoms of sepsis and has a clinical presentation ranging from confusion to delirium and coma. The symptom affects up to 70% of patients with sepsis (15) and increases the mortality rate (12). In the study by Krzyżaniak *et al* (12), which included 443 patients with sepsis, it was reported that 240 patients developed SAE; the mortality rate in the SAE group (45.42%) was significantly higher than that in the non-SAE group (7.88%). The liver plays a key role in immune responses through the synthesis of acute-phase proteins and complement components, cytokine production and pathogen clearance via Kupffer cells, functioning as a critical immunometabolic defense organ (16). The presence of chronic comorbidities alongside sepsis markedly increases the risk of end-organ failure (16,17). Therefore, in the treatment of sepsis, it is important to implement therapeutic strategies not only targeting the infectious agent, but also addressing underlying chronic conditions (14,17).

With the rise of sedentary lifestyles and obesity, diabetes has become a major global metabolic disease, currently affecting ~537 million adults worldwide and projected to reach 783 million by 2045 (18). Diabetes is accompanied by chronic hyperglycemia, which increases the production of reactive oxygen species (ROS) within the cell (19). These radicals cause oxidative stress, leading to damage to cell membranes, proteins and DNA. Oxidative stress serves a role in the development of diabetic complications such as neuropathy, retinopathy and nephropathy (20,21). For this reason, antioxidant therapies are being investigated to reduce the oxidative damage associated with diabetes (20). In previous years, nanomaterials exhibiting antioxidant properties, particularly carbon-based nanomaterials, have emerged as potential therapeutic agents in this context (21). In the management of diabetes, reducing oxidative stress is considered as important as controlling blood glucose levels (19-21).

In diabetes, mitochondrial dysfunction has been observed; however, the underlying molecular mechanisms have not been fully elucidated (22), despite it contributing towards inflammatory processes by driving increased anaerobic metabolism at the tissue level, thereby increasing sepsis-related mortality (23). Sepsis-induced heart and kidney damage is a frequent complication in intensive care units and leads to high morbidity and mortality (24). The liver is an organ that serves a vital role in functions such as detoxification, immune regulation and the antioxidant system (including glutathione production) of the body (25). Therefore, protecting the liver during sepsis strengthens the antioxidant capacity of the body. In sepsis, increased blood-brain barrier permeability, overproduction of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), IL-1 β and IL-6, their infiltration into the brain parenchyma, activation of glial cells and sepsis-related mitochondrial dysfunction lead to an enhanced neuroimmune response, resulting in neuronal cell death (23).

In diabetic mice, fullereneol C₆₀, with its notable antioxidant capacity, has been shown to protect against ischemia-reperfusion (I/R) injury in muscle, heart and lung tissues (26,27). Yavuz *et al* (28) also observed the protective effects of fullereneol C₆₀ on liver tissue in a liver I/R injury model. Although very few studies have demonstrated the effectiveness of fullereneol C₆₀ in sepsis, in our previous study, its protective effects on the kidneys and lungs were demonstrated in an abdominal sepsis model (29). Furthermore, the present study aimed to prevent multiple organ failure that may develop due to sepsis in diabetic rats by utilizing the antioxidant properties of fullereneol C₆₀, as well as investigate its protective effects on the heart, brain and liver.

Materials and methods

In accordance with ARRIVE 2.0 guidelines (30), the present study was conducted at The Gazi University Laboratory Animal Breeding and Experimental Research Center (Ankara, Turkey) with approval from The Gazi University Experimental Animal Ethics Committee (Ankara, Turkey; approval no. G.Ü.ET-24.046). The present study was also conducted in accordance with The Guide for the Care and Use of Laboratory Animals (31).

A total of 30 Wistar albino male rats (6-7 weeks old; body weight, 200-225 g), obtained from the Gazi University Laboratory Animal Breeding and Experimental Research Center (Ankara, Türkiye), were used, comprising 24 rats with streptozotocin-induced diabetes and 6 healthy rats that served as the control group. The number of rats was determined according to the maximum number of rats permitted by the ethics committee. The rats were housed under a 12 h light/dark cycle, with an environmental temperature of 20-21°C and humidity of 45-55%. The rats had free access to food and water.

Induction of diabetes. To induce diabetes, the same procedures were applied as outlined in our previous studies (32,33).

Experimental design. Rats were randomly assigned to four groups: Six in the control group and eight in all other groups. Before laparotomy, all rats were anesthetized through intramuscular administration of ketamine (50 mg/kg; Ketalar®; Pfizer Türkiye) and xylazine hydrochloride (10 mg/kg; Dogalazin® 2% xylazine injectable solution; Doğa İlaç). Procedures were performed under a heating lamp with the rats in the supine position to prevent heat loss during the experiment. The rats were treated as follows: i) In the control (S) group (n=6), after anesthesia, rats underwent midline laparotomy and the abdomen was closed without further intervention; ii) in the diabetic control (D) group (n=8), rats with streptozotocin-induced diabetes underwent the same sham laparotomy procedure as the S group without CLP induction; iii) in the diabetes + CLP (DM/SEP) group (n=8), rats underwent midline laparotomy followed by cecal ligation and puncture (CLP), performed using a 21-gauge syringe needle at the antimesenteric border, 1-2 cm distal to the ileocecal valve. The cecum was repositioned, and the abdomen was closed; iv) in the diabetes + CLP + fullereneol C₆₀ (FUL/C-60) group (n=8), rats underwent the same CLP procedure as the DM/SEP group. At 30 min post-abdominal closure, fullereneol C₆₀ (100 mg/kg) was administered intraperitoneally. The route of administration and dosage were determined based on previous studies (26-29). At 24 h post-laparotomy, the rats were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg), and sacrificed via intracardiac blood collection (5-10 ml). After cessation of heartbeat and respiration, animals were monitored for an additional 2 min. Liver, heart and brain tissues were carefully harvested for histopathological and oxidative stress analyses, and blood samples were collected for biochemical evaluation. No rats were lost during the study. Pre-determined humane endpoints were established in accordance with institutional animal care guidelines and included severe lethargy, inability to access food or water, persistent hypothermia, loss of >20% of body weight or signs of severe distress; animals meeting any of these criteria were humanely euthanized.

Histopathological evaluation. Liver, heart and brain tissues were fixed in 10% neutral-buffered formalin at room temperature (20-25°C) for 24 h and embedded in paraffin. Paraffin blocks were sectioned at a 4-μm thickness using a rotary microtome. After deparaffinization in xylene and rehydration

through graded ethanol, sections were stained with hematoxylin (5 min) and eosin (2 min) at room temperature. These sections were two experienced histopathologists, blinded to the procedures, using a semiquantitative grading scale (0, no change; +1, minimal change; +2, moderate change; +3, severe change) (29).

Biochemical evaluation. Oxidative stress assessment in the brain, liver and heart tissue samples was performed by measuring catalase (CAT) activity and thiobarbituric acid reactive substance (TBARS) levels. The TBARS assay for assessing lipid peroxidation was performed using the TBA method. Tissue homogenates were mixed with trichloroacetic acid and TBA solution and heated in a boiling water bath (95-100°C) for 15 min under acidic conditions. After cooling, the mixture was centrifuged at 1,600 x g for 10 min at 4°C, and the absorbance of the supernatant was measured spectrophotometrically at 532 nm. Malondialdehyde levels were calculated using 1,1,3,3-tetraethoxypropane as the standard (MilliporeSigma). CAT activity was measured based on the decomposition of hydrogen peroxide (H₂O₂). The decrease in absorbance due to H₂O₂ consumption was monitored at 240 nm for 1 min at room temperature using a spectrophotometer, and enzyme activity was expressed as U/mg protein. For serum biochemical analysis, blood samples were centrifuged at 1,000 x g for 10 min at room temperature to obtain serum. Serum AST (cat. no. 04490734190), ALT (cat. no. 04490718190), γ-GGT (cat. no. 03002728122), total bilirubin (cat. no. 05795393190), direct bilirubin (cat. no. 05795423190) and albumin (cat. no. 05166861190) levels were measured using commercially available assay kits (Roche Diagnostics) on an automated biochemical analyzer according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS for Windows (version 26.0; IBM Corp.) employing the tests listed below. Data distribution was analyzed using the Shapiro-Wilk test. Comparisons of >2 groups were performed using the Kruskal-Wallis test followed by Dunn's post hoc test or one-way ANOVA followed by Tukey's post hoc test, as appropriate. P<0.05 was considered to indicate a statistically significant difference. Data are expressed as mean ± SD and median with interquartile range.

Results

Histopathological examination of liver tissue. Under light microscopy, hepatocyte degeneration differed significantly among groups (P=0.017), being more frequent in the DM/SEP group compared with the S and D groups (P=0.001 and P=0.013, respectively). However, it was significantly reduced in the FUL/C-60 group compared with that in the DM/SEP group (P=0.042) (Table I; Figs. 1-4).

Sinusoidal dilatation also differed significantly among groups (P=0.022), being more prominent in the DM/SEP group compared with the S group (P=0.002) and significantly lower in the FUL/C-60 group compared with the DM/SEP group (P=0.013) (Table I; Figs. 1-4).

The presence of pyknotic cells differed significantly among groups (P=0.044). Pyknotic cells were more commonly

Table I. Histopathological findings of the S group (n=6), D group (n=8), DM/SEP group (n=8) and FUL/C-60 group (n=8) in liver tissue.

Feature	S group	D group	DM/SEP	FUL/C-60	P-value
Hepatocyte degeneration	0.50 (0.00-1.00)	1.00 (0.75-1.00)	1.50 (1.00-2.25) ^{a,b}	1.00 (1.00-1.00) ^c	0.017
Sinusoidal dilatation	0.00 (0.00-1.00)	1.00 (0.75-1.25)	1.50 (1.00-2.25) ^a	1.00 (0.00-1.00) ^c	0.022
Pyknotic cells	0.00 (0.00-1.00)	0.50 (0.00-1.00)	1.00 (1.00-2.00) ^{a,b}	0.50 (0.00-1.00) ^c	0.044
Cells undergoing necrosis	0.00 (0.00-0.25)	0.50 (0.00-1.00)	1.50 (1.00-2.25) ^{a,b}	1.00 (1.00-1.00) ^{a,c}	<0.001
Parenchymal MN cell infiltration	0.00 (0.00-1.00)	1.00 (0.00-1.25)	1.00 (1.00-2.00) ^a	1.00 (1.00-1.00) ^a	0.035

All values are presented as the median (IQR). P-values were determined by Kruskal-Wallis tests followed by Dunn's post hoc test ($P < 0.05$).

^a $P < 0.05$ compared with the S group, ^b $P < 0.05$ compared with the D group and ^c $P < 0.05$ compared with the DM/SEP group. S, control; D, diabetic control; DM/SEP, diabetes + sepsis; FUL/C-60, diabetes + sepsis + fullerenol C₆₀; MN, mononuclear.

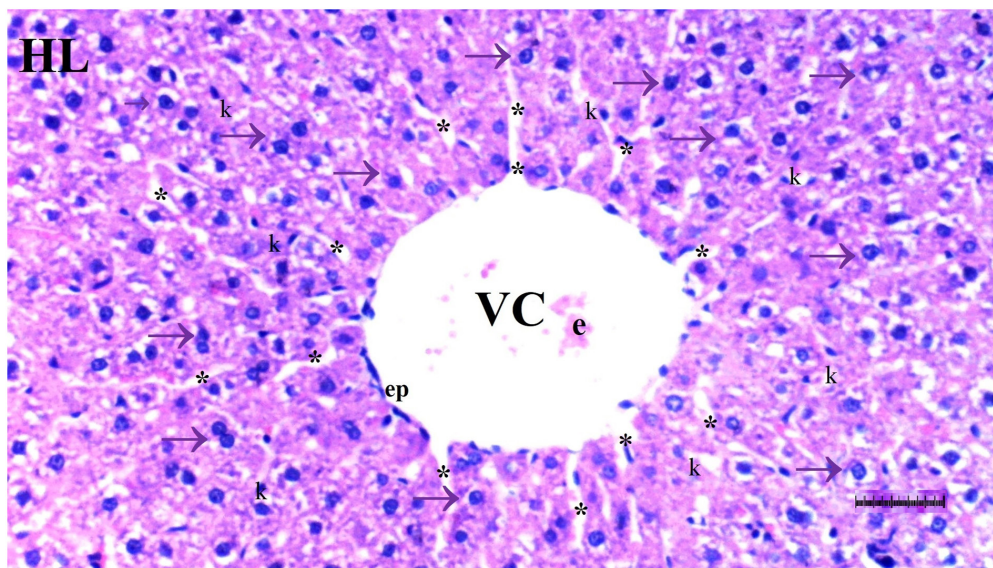


Figure 1. Representative light microscopy of liver tissue from the group control group. H&E staining. Magnification, x100. Arrows represent hepatocytes. VC, vena centralis; HL, hepatic lobule; e, erythrocyte; ep, epithelium; k, Kupffer cells; *, sinusoids.

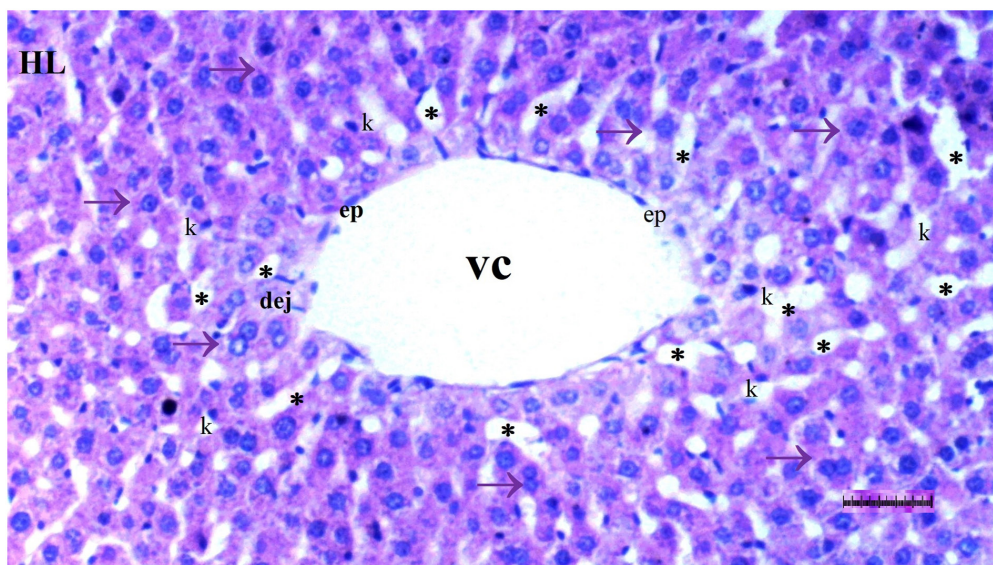


Figure 2. Representative light microscopy of liver tissue from the diabetic control group. H&E staining. Magnification, x100. Arrows represent hepatocytes. vc, vena centralis; HL, hepatic lobule; dej, hepatocyte degeneration; ep, epithelium; k, Kupffer cells; *, sinusoids.

Table II. Histopathological findings of the S group (n=6), D group (n=8), DM/SEP group (n=8) and FUL/C-60 group (n=8) in heart tissue.

Feature	S group	D group	DM/SEP	FUL/C-60	P-value
Inflammation	0.00 (0.00-0.25)	0.50 (0.00-1.00)	1.00 (1.00-1.00) ^a	0.50 (0.00-1.00)	0.036
Myocardial disorganization	0.50 (0.00-1.00)	1.00 (0.75-1.00)	1.00 (1.00-2.00)	1.00 (0.75-1.00)	0.068
Interstitial fibrosis	0.00 (0.00-1.00)	0.50 (0.00-1.00)	1.00 (1.00-2.00) ^{a,b}	0.50 (0.00-1.00) ^c	0.040

All values are presented as the median (IQR). P-values were determined by Kruskal-Wallis tests followed by Dunn's post hoc test ($P < 0.05$). ^a $P < 0.05$ compared with the S group, ^b $P < 0.05$ compared with the D group and ^c $P < 0.05$ compared with the DM/SEP group. S, control; D, diabetic control; DM/SEP, diabetes + sepsis; FUL/C-60, diabetes + sepsis + fullereneol C₆₀.

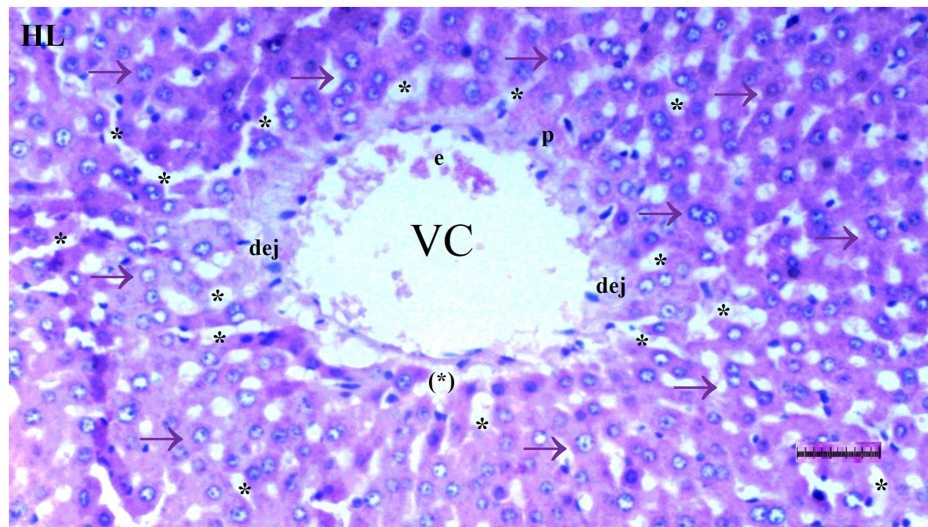


Figure 3. Representative light microscopy of liver tissue from the diabetes + sepsis group. H&E staining. Magnification, x100. Arrows represent hepatocytes. VC, vena centralis; HL, hepatic lobule; dej, hepatocyte degeneration; e, erythrocyte; p, picnotic and hyperchromatic cells; k, Kupffer cells; *, sinusoids; (*), sinusoid dilatation.

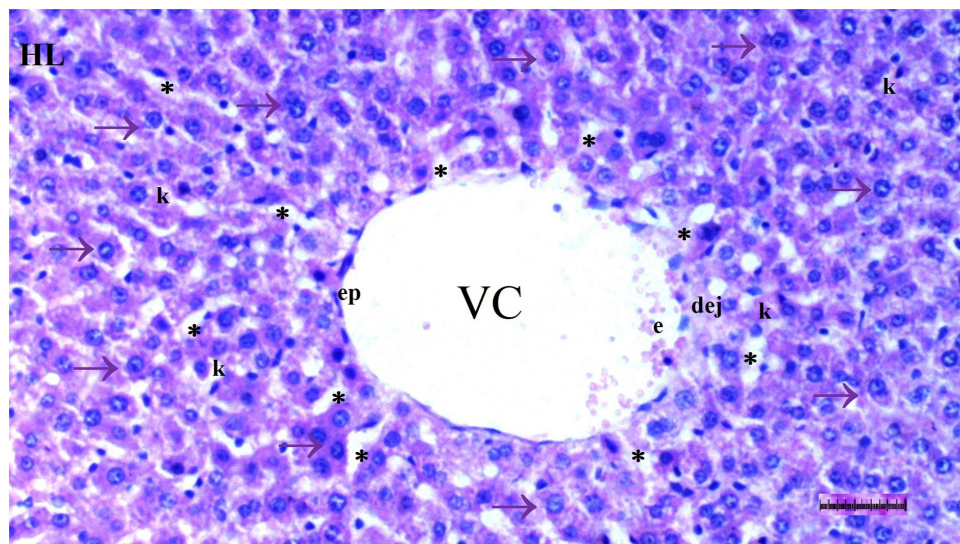


Figure 4. Representative light microscopy of liver tissue from the diabetes + sepsis + fullereneol C₆₀ group. H&E staining. Magnification, x100. Arrows represent hepatocytes. VC, vena centralis; HL, hepatic lobule; dej, hepatocyte degeneration; ep, epithelium; e, erythrocyte; k, Kupffer cells; *, sinusoids.

observed in the DM/SEP group compared with the S and D groups ($P = 0.003$ and $P = 0.037$, respectively), whereas pyknotic

cells were significantly fewer in the FUL/C-60 group compared with the DM/SEP group ($P = 0.037$) (Table I; Figs. 1-4).

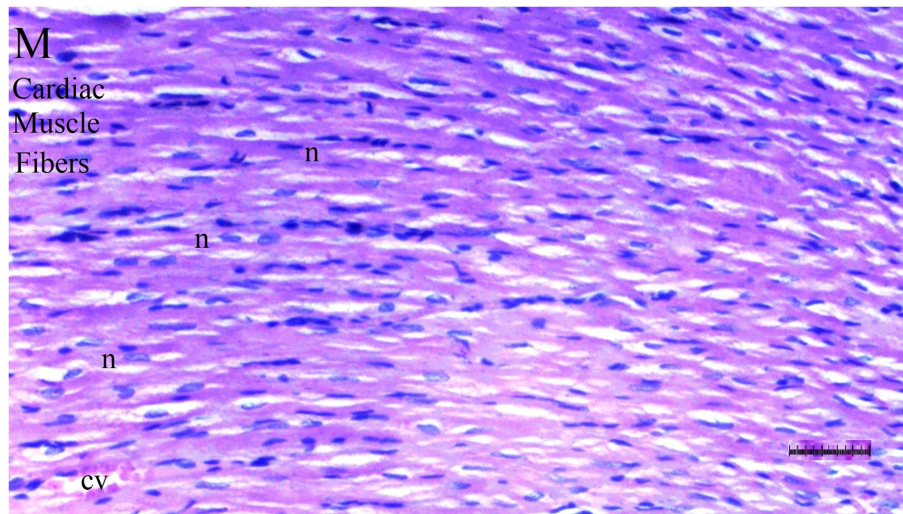


Figure 5. Representative light microscopy of heart tissue from the control group. H&E staining. Magnification, x100. M, myocardium; n, nuclei; cv, cardiac blood vessel.

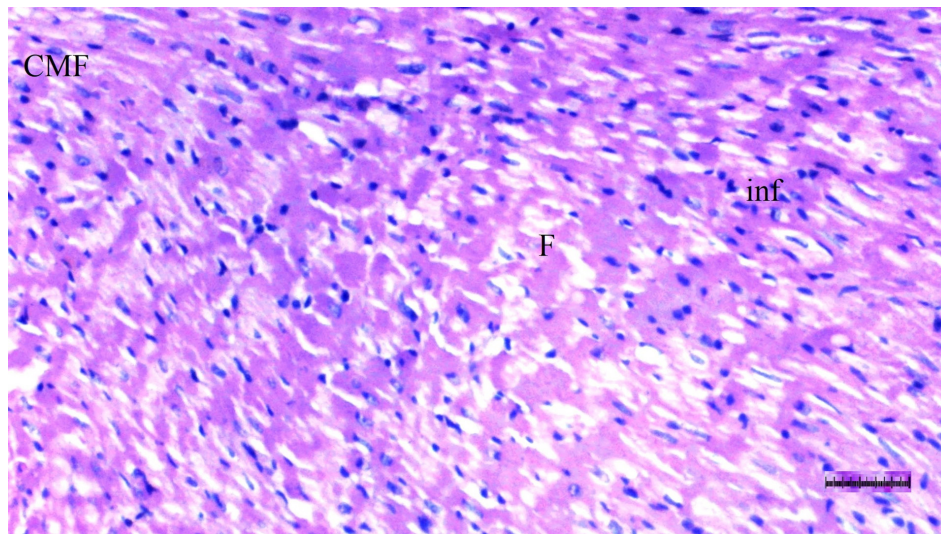


Figure 6. Representative light microscopy of heart tissue from the diabetic control group. H&E staining. Magnification, x100. CMF, cardiac muscle fibers; F, fibrosis; inf, inflammation.

The number of cells undergoing necrosis differed significantly among groups ($P < 0.001$). More necrotic cells were observed in the DM/SEP group compared with the S and D groups ($P < 0.001$ and $P = 0.004$, respectively). Furthermore, the number of necrotic cells was higher in the FUL/C-60 group compared with the S group ($P = 0.012$), but significantly lower in the FUL/C-60 group compared with the DM/SEP group ($P = 0.040$) (Table I; Figs. 1-4).

Mononuclear (MN) cell infiltration in the parenchyma was significantly different among groups ($P = 0.035$). MN cell infiltration was more pronounced in the DM/SEP and FUL/C-60 groups compared with the S group ($P = 0.004$ and $P = 0.040$, respectively) (Table I; Figs. 1-4).

Histopathological examination of heart tissue. Inflammation varied significantly among groups ($P = 0.036$). The DM/SEP group exhibited a significantly higher degree of inflammation compared with the S group ($P = 0.013$) (Table II; Figs. 5-8).

Interstitial fibrosis differed significantly among groups ($P = 0.040$); it was significantly greater in the DM/SEP group compared with the S and D groups ($P = 0.004$ and $P = 0.012$, respectively); however, it was significantly reduced in the FUL/C-60 group compared with the DM/SEP group ($P = 0.040$) (Table II; Figs. 5-8).

Histopathological examination of brain tissue. An increase in cellularity (macrophages and astrocytes) was found to be significantly different among groups ($P = 0.014$). The DM/SEP group showed a greater cellularity compared with the S and D groups ($P < 0.001$ and $P = 0.003$, respectively), while the FUL/C-60 group exhibited significantly lower cellularity (macrophages and astrocytes) compared with the DM/SEP group ($P = 0.034$) (Table III; Figs. 9-12). Inflammation, both macrophagic and astroglial, was significantly different among groups ($P = 0.036$), being significantly more pronounced in the DM/SEP group compared with the S group ($P = 0.003$).

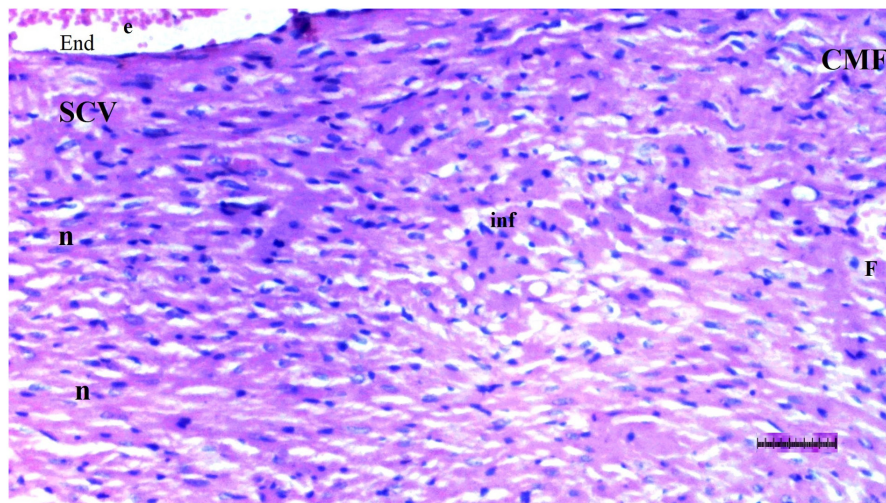


Figure 7. Representative light microscopy of heart tissue from the diabetes + sepsis group. H&E staining. Magnification, x100. CMF, cardiac muscle fibers; F, fibrosis; inf, inflammation; e, erythrocyte; End, endothelium.

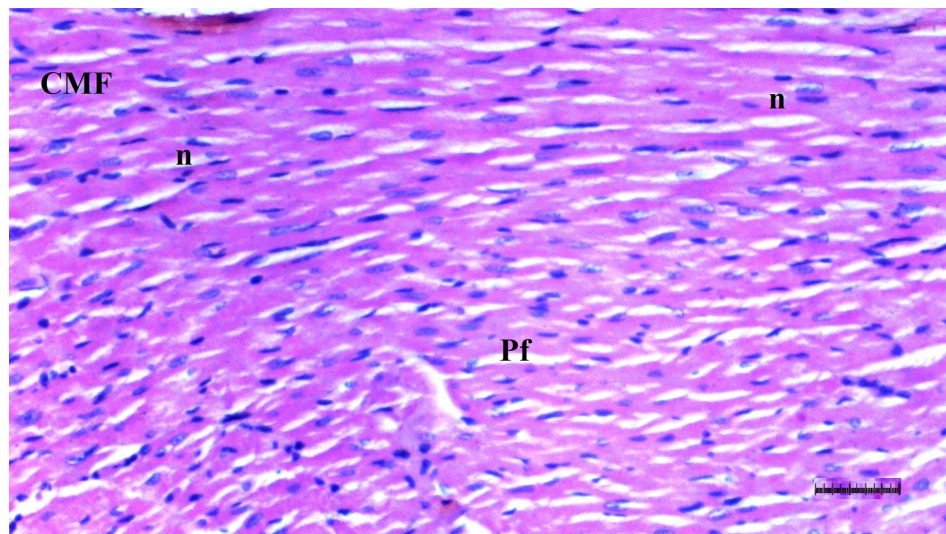


Figure 8. Representative light microscopy of heart tissue from the diabetes + sepsis + fullereneol C₆₀ group. H&E staining. Magnification, x100. CMF, cardiac muscle fibers; Pf, Purkinje muscle fibers; n, nuclei.

Liver tissue oxidant status parameter assessment. When liver tissue was compared in terms of TBARS levels, a significant difference was observed among groups ($P < 0.001$). TBARS levels were significantly higher in all groups compared with the S group (all $P < 0.001$) and in the DM/SEP and FUL/C-60 groups compared with the D group ($P < 0.001$ and $P = 0.002$, respectively). These levels were significantly lower in the FUL/C-60 group compared with the DM/SEP group ($P < 0.001$) (Table IV).

With regard to CAT enzyme activity in liver tissue, a significant difference was observed among groups ($P < 0.001$). CAT enzyme activity was significantly lower in all experimental groups compared with the S group ($P = 0.004$, $P < 0.001$, and $P < 0.001$, respectively) and in the DM/SEP and FUL/C-60 groups compared with the D group (both $P < 0.001$). CAT activity was significantly higher in the FUL/C-60 group compared with the DM/SEP group ($P = 0.034$) (Table IV).

Brain tissue oxidant status parameter assessment. When brain tissue was compared in terms of TBARS levels, a significant difference was identified ($P < 0.001$). TBARS levels were significantly higher in all groups compared with the S group (all, $P < 0.001$). In addition, TBARS levels were significantly higher in the DM/SEP group compared with the D group ($P < 0.001$) and significantly lower in the FUL/C-60 group compared with the DM/SEP group ($P < 0.001$) (Table V). With regard to CAT enzyme activity in brain tissue, a significant difference was observed among groups ($P < 0.001$). Levels were significantly lower in the DM/SEP and FUL/C-60 groups compared with the D group (both, $P < 0.001$) and significantly higher in the FUL/C-60 group compared with the DM/SEP group ($P = 0.009$; Table V).

Serum AST, ALT, GGT, total bilirubin, direct bilirubin and albumin results. Serum AST, ALT, total bilirubin, direct bilirubin and albumin levels differed significantly

Table III. Histopathological findings of the S group (n=6), D group (n=8), DM/SEP group (n=8) and FUL/C-60 group (n=8) in brain-cerebellar tissue.

Feature	S group	D group	DM/SEP	FUL/C-60	P-value
Ischemic neuronal change (cortex/medulla)	0.00 (0.00-0.25)	0.00 (0.00-1.00)	0.50 (0.00-1.25)	0.00 (0.00-1.00)	0.614
Increased cellularity (macrophages and astrocytes)	0.00 (0.00-1.00)	0.50 (0.00-1.00)	1.50 (1.00-2.00) ^{a,b}	1.00 (0.00-1.00) ^c	0.014
Focal necrosis	0.00 (0.00-1.00)	0.00 (0.00-1.00)	0.50 (0.00-1.25)	0.50 (0.00-1.00)	0.819
Inflammation (macrophagic and astroglial)	0.00 (0.00-0.25)	0.50 (0.00-1.00)	1.00 (1.00-1.00) ^a	0.50 (0.00-1.00)	0.036

All values are presented as the median (IQR). P-values were determined by Kruskal-Wallis tests followed by Dunn's post hoc test ($P < 0.05$).

^a $P < 0.05$ compared with the S group, ^b $P < 0.05$ compared with the D group and ^c $P < 0.05$ compared with the DM/SEP group. S, control; D, diabetic control; DM/SEP, diabetes + sepsis; FUL/C-60, diabetes + sepsis + fullerenol C₆₀.

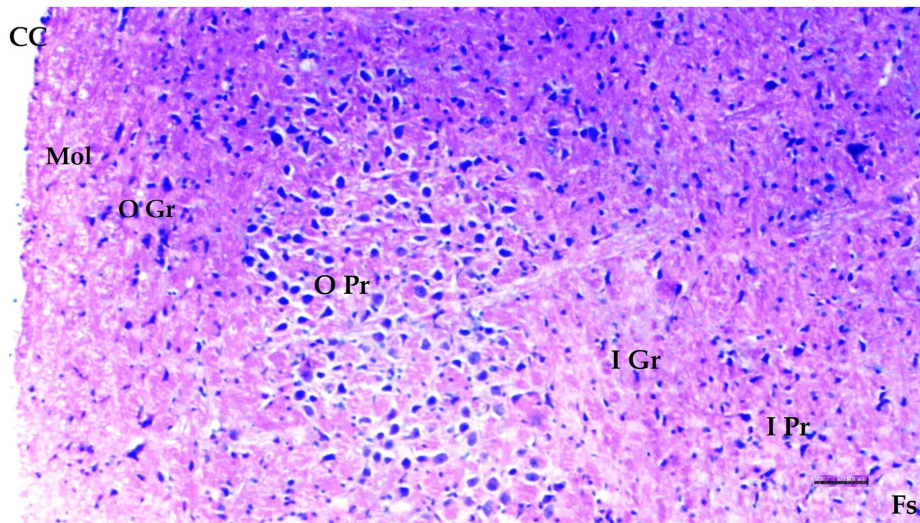


Figure 9. Representative light microscopy of brain tissue from the control group. H&E staining. Magnification, x40. CC, cerebral cortex; Mol, molecular layer; O Gr, outer granular layer; O Pr, outer pyramidal layer; I Gr, inner granular layer; I Pr, inner pyramidal layer; Fs, fusiform layer.

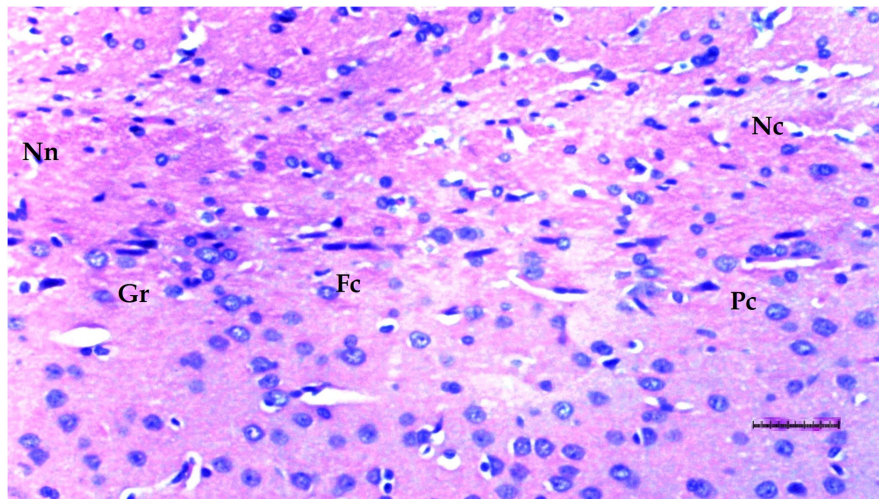


Figure 10. Representative light microscopy of brain tissue from the diabetic control group. H&E staining. Magnification, x100. Nc, neuroglial cells; Nn, neuroglia nuclei; Fc, fusiform cells; Pc, pyramidal cells; Gr, granular layer.

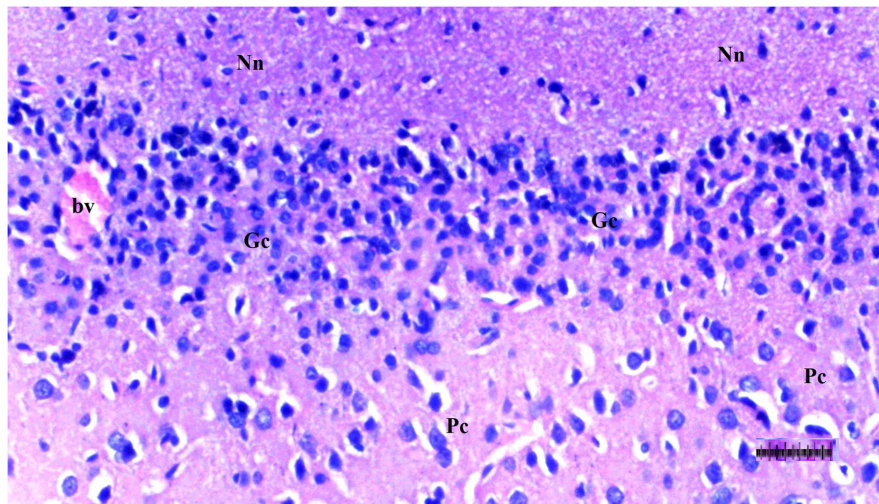


Figure 11. Representative light microscopy of brain tissue from the diabetes + sepsis group. H&E staining. Magnification, x100. Nn, neuroglia nuclei; Gc, fusiform cells; Pc, pyramidal cells; bv, blood vessels.

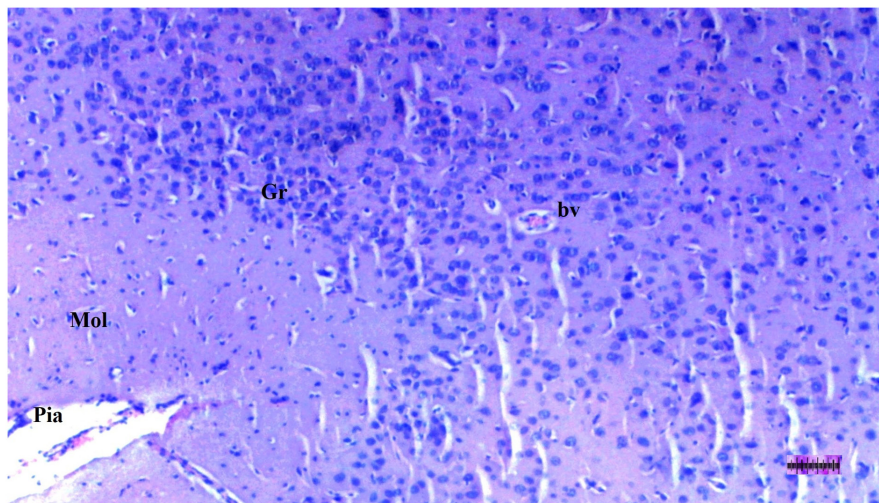


Figure 12. Representative light microscopy of brain tissue from the diabetes + sepsis + fullereneol C₆₀ group. H&E staining. Magnification, x40. Mol, molecular layer; Gr, granular layer; Pia, pia matter; bv, blood vessels.

among groups (all $P < 0.001$; Table VI). AST and ALT levels were significantly elevated in the DM/SEP and FUL/C-60 groups compared with the S (all $P < 0.001$) and D groups (all $P < 0.001$). Levels were significantly lower in the FUL/C-60 group compared with the DM/SEP group (both $P < 0.001$) (Table VI). GGT levels were significantly higher in the DM/SEP and FUL/C-60 groups compared with the S group ($P < 0.001$ and $P = 0.004$, respectively) and the D group ($P < 0.001$ and $P = 0.032$, respectively). Levels were significantly lower in the FUL/C-60 group compared with the DM/SEP group ($P = 0.008$) (Table VI). Total and direct bilirubin levels were significantly higher in the DM/SEP and FUL/C-60 groups compared with the S group (all $P < 0.001$) but significantly lower in the FUL/C-60 group compared with the DM/SEP group (both $P < 0.001$) (Table VI). Albumin levels were significantly lower in the DM/SEP and FUL/C-60 groups compared with the S group ($P < 0.001$ and $P = 0.005$, respectively) and the D group ($P = 0.004$) (Table VI).

Discussion

In the present study, the histopathological and biochemical effects of fullereneol C₆₀ on sepsis-induced multiple organ failure were evaluated in a diabetic rat model of sepsis. The findings obtained through light microscopy and biochemical analyses demonstrated that pathological alterations triggered by sepsis were markedly increased, particularly in the liver, heart and brain tissues and that the administration of fullereneol C₆₀ significantly attenuated these changes.

A number of antioxidant agents have been investigated in diabetic or septic models with varying degrees of success, including melatonin. Taher *et al* (34) reported that high-dose melatonin (50 mg for 5 consecutive nights) administered to patients with early septic shock was associated with a reduction in the Sequential Organ Failure Assessment (SOFA) score (11), a commonly used measure of organ dysfunction severity in critically ill patients, with higher scores being associated with increased mortality. Compared with placebo,

Table IV. Oxidative status parameters of the S group (n=6), D group (n=8), DM/SEP group (n=8) and FUL/C-60 group (n=8) in liver tissue.

Parameter	S group	D group	DM/SEP	FUL/C-60	P-value
TBARS, nmol/mg	1.20±0.08	2.02±0.09 ^a	4.71±0.39 ^{a,b}	2.63±0.44 ^{a,b,c}	<0.001
CAT, IU/mg	422.88±38.12	312.88±106.76 ^a	98.28±9.17 ^{a,b}	175.70±29.62 ^{a,b,c}	<0.001

All values are presented as the mean ± SD. P-values were determined by ANOVA tests followed by Tukey's post hoc test (P<0.05). ^aP<0.05 compared with the S group, ^bP<0.05 compared with the D group and ^cP<0.05 compared with the DM/SEP group. S, control; D, diabetic control; DM/SEP, diabetes + sepsis; FUL/C-60, diabetes + sepsis + fullereneol C₆₀; TBARS, thiobarbituric acid reactive substance; CAT, catalase.

Table V. Oxidative status parameters of the S group (n=6), D group (n=8), DM/SEP group (n=8) and FUL/C-60 group (n=8) in brain tissue.

Parameter	S group	D group	DM/SEP	FUL/C-60	P-value
TBARS, nmol/mg	1.57±0.30	2.41±0.28 ^a	4.29±0.38 ^{a,b}	2.67±0.37 ^{a,b,c}	<0.001
CAT, IU/mg	403.28±35.09	278.28±61.88 ^a	91.25±13.36 ^{a,b}	156.75±30.60 ^{a,b,c}	<0.001

All values are presented as the mean ± SD. P-values were determined by ANOVA tests followed by Tukey's post hoc test (P<0.05). ^aP<0.05 compared with the S group, ^bP<0.05 compared with the D group and ^cP<0.05 compared with the DM/SEP group. S, control; D, diabetic control; DM/SEP, diabetes + sepsis; FUL/C-60, diabetes + sepsis + fullereneol C₆₀; TBARS, thiobarbituric acid reactive substance; CAT, catalase.

Table VI. Serum levels of AST, ALT, GGT, bilirubin and albumin in the S group (n=6), D group (n=8), DM/SEP group (n=8) and FUL/C-60 group (n=8).

Parameter	S group	D group	DM/SEP	FUL/C-60	P-value
AST, IU/l	141.17±8.90	171.00±25.04	1,944.50±313.69 ^{a,b}	1,133.17±34.36 ^{a,b,c}	<0.001
ALT, IU/l	65.33±13.87	66.83±12.92	305.83±55.28 ^{a,b}	210.66±44.98 ^{a,b,c}	<0.001
GGT, U/l	6.50±0.55	7.67±1.63	14.50±2.88 ^{a,b}	10.67±3.01 ^{a,b,c}	<0.001
Total bilirubin, mg/dl	0.11±0.01	0.12±0.03	0.34±0.09 ^{a,b}	0.14±0.04 ^c	<0.001
Direct bilirubin, mg/dl	0.10±0.00	0.12±0.04	0.29±0.09 ^{a,b}	0.12±0.03 ^c	<0.001
Albumin, g/dl	3.80±0.41	3.53±0.49	2.85±0.22 ^{a,b}	3.15±0.24 ^a	<0.001

All values are presented as the mean ± SD. P-values were determined by ANOVA tests followed by Tukey's post hoc test (P<0.05). ^aP<0.05 compared with the S group, ^bP<0.05 compared with the D group and ^cP<0.05 compared with the DM/SEP group. S, control; D, diabetic control; DM/SEP, diabetes + sepsis; FUL/C-60, diabetes + sepsis + fullereneol C₆₀; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

melatonin treatment also resulted in a greater proportion of patients achieving SOFA scores ≤6 by day 28, along with markedly increased ventilator-free and vasopressor-free days, suggesting a potential role for melatonin as an adjunctive therapy in septic shock.

Rosengrave *et al* (35) evaluated the effects of intravenous vitamin C (25 mg/kg every 6 h for up to 96 h) on vasopressor requirements in 40 patients with septic shock. The study found no significant differences between the vitamin C and placebo groups in terms of vasopressor dose or duration, SOFA score, intensive care unit or hospital length of stay, or mortality, and stated that further studies are needed in this area. In their study evaluating the effects of curcumin supplementation (1,000 mg daily for 12 weeks) on psychological status and markers of inflammation and oxidative stress in patients with type 2

diabetes and coronary heart disease, Shafabakhsh *et al* (36) observed that curcumin intake markedly improved sleep quality and reduced oxidative damage, with a decrease in malondialdehyde levels and an increase in total antioxidant capacity and glutathione levels. These findings demonstrated that curcumin provides systemic antioxidant and anti-inflammatory benefits in patients with diabetes and cardiovascular disease. In line with the literature, the present results showed that fullereneol C₆₀, reported to possess potent free radical scavenging and antioxidant properties (37), significantly reduced TBARS levels in liver and brain tissues and increased CAT activity. Although oxidative stress markers (TBARS and CAT) were evaluated only in liver and brain tissues, not in cardiac tissue, in the present study, significant histopathological improvement was observed in the liver, brain and heart.

Fullerenol C₆₀, reported to possess potent antioxidant and free radical scavenging properties (37), can readily react with radical species through conjugated carbon-carbon double bonds, neutralizing oxygen-free radicals and mimicking superoxide dismutase (SOD) activity. The transcription factor nuclear factor erythroid 2 p45-associated factors 2 (Nrf2), regulates the endogenous antioxidant response pathway by binding to antioxidant response elements (AREs) in the promoter regions of antioxidants. Nrf2 regulates glutathione (GSH) recycling by controlling the expression of γ -GCL, a key enzyme in GSH biosynthesis. The matrix GSH redox cycle, coordinated with SOD-mediated clearance of superoxide anion, is key in preventing excessive ROS accumulation in the mitochondria. C₆₀ has also been shown to modulate the activity and expression of antioxidant enzymes such as SOD and GSH through Nrf2/ARE-dependent pathways (37).

Sepsis is a major contributor to multiple organ failure, particularly through structural and functional impairments in the liver. Systemic inflammatory response-induced hypoperfusion and impaired oxygenation result in mitochondrial dysfunction and cellular injury in hepatocytes (38). This process increases ROS, which, in turn, leads to an increase in oxidative stress. Increased oxidative stress leads to lipid peroxidation in hepatocyte membranes, accelerating the hepatocyte injury and cell death. These alterations impair hepatic detoxification and bile excretory function, potentially leading to cholestasis and elevated bilirubin levels (38,39).

Furthermore, under the influence of pro-inflammatory cytokines, such as TNF- α , interleukin (IL)-1 β and IL-6, hepatic microcirculation is disrupted, leading to a marked increase in hepatic enzyme levels. Consequently, sepsis-related liver dysfunction exacerbates disease severity and worsens prognosis (39). In addition, the systemic inflammatory response may induce notable dysfunction in the cardiovascular system. Increased vasodilation and capillary permeability lead to hypotension and impaired tissue perfusion. Mitochondrial dysfunction and cellular damage in cardiomyocytes, driven by inflammatory mediators, result in myocardial depression and reduced cardiac output. In addition, endothelial dysfunction and coagulopathy impair microcirculation by increasing vascular permeability and promoting microthrombus formation, thereby reducing capillary blood flow and leading to tissue hypoperfusion. These mechanisms form the basis of the high mortality rates associated with cardiovascular complications in sepsis (40).

Furthermore, sepsis can affect the central nervous system and lead to the development of sepsis-associated encephalopathy, a marked neurological complication that contributes to increased morbidity and mortality. Systemic inflammatory response and cerebral hypoperfusion induce metabolic stress in neurons, characterized by mitochondrial dysfunction and reduced ATP levels. During this pathophysiological process, excessive ROS production triggers oxidative damage, leading to lipid peroxidation, protein denaturation and disruption of DNA integrity. Furthermore, increased blood-brain barrier permeability facilitates the passage of peripheral pro-inflammatory cytokines, such as TNF- α , interleukin (IL)-1 β and IL-6, into the brain parenchyma, enhancing glial activation and amplifying the neuroinflammatory response (15). Consequently, neuronal dysfunction and cellular injury

provide the groundwork for a clinical setting characterized by cognitive impairment, confusion and decreased levels of consciousness.

In the present study, histopathological findings in line with those reported by Elshater *et al* (41) were observed. This study investigated the effects of fullerenes on the liver in rats. In particular, the notably increased level of hydropic degeneration, sinusoidal dilatation, pyknotic cells and necrotic cells in the DM/SEP group reflected the extent of liver damage induced by sepsis in diabetic rats. Sepsis-induced oxidative stress leads to the generation of free radicals that disrupt cellular functions and contribute to cell death. The reduction of these pathological changes in the FUL/C-60 group highlights the ability of fullereneol C₆₀ to counteract free radical-induced cellular damage through its antioxidant properties.

The effect of fullereneol C₆₀ on hepatic oxidative stress was also evident in the present biochemical analyses. TBARS levels, an indicator of lipid peroxidation and oxidative stress, are of particular importance. In the present study, findings similar to those in previous work by Kubat *et al* (29) were observed, a study in which the impact of fullereneol C₆₀ on TBARS levels in kidney and lung tissues was reported. Similarly, Yavuz *et al* (28) demonstrated marked changes in TBARS levels associated with the effect of fullereneol C₆₀ on the liver. In the present study, the significantly elevated TBARS levels in the DM/SEP group reflected the oxidative stress caused by sepsis, while the reduction of this parameter in the FUL/C-60 group suggested that fullereneol C₆₀ mitigated lipid peroxidation and limited oxidative tissue damage. Furthermore, the increase in CAT enzyme activity indicated the potential of fullereneol C₆₀ to support endogenous antioxidant defense mechanisms. This finding implied that fullereneol C₆₀ modulated endogenous antioxidant enzyme activity and reduced the impact of free radicals.

As previously reported (41-46), the beneficial effects of fullerenes on the liver are well established. In the present study, significantly elevated levels of liver injury markers such as AST, ALT, GGT and bilirubin in the DM/SEP group indicated hepatic damage and leakage of intracellular enzymes into serum due to sepsis. However, the reduction in these enzyme levels in the FUL/C-60 group suggested that fullereneol C₆₀ may exert hepatoprotective effects by maintaining hepatocyte stability and limiting enzyme release, findings that align with previous studies (41-46). This also indicates the potential anti-inflammatory properties of fullereneol C₆₀, as suppression of sepsis-related inflammatory processes is key in preventing multiple organ failure.

Sepsis not only affects the liver but also negatively impacts cardiac tissue. In the present study, cardiac oxidative stress markers (such as TBARS and CAT) and inflammatory mediators were not assessed in heart tissue. However, histopathological findings showed that myocardial disorganization and interstitial fibrosis were more pronounced in the DM/SEP group compared with the other groups, indicating the detrimental cardiac effects of sepsis in patients with diabetic. Sepsis triggers a complex interplay of immune activation, oxidative stress, mitochondrial failure and cardiomyocyte apoptosis, resulting in myocardial disorganization and disruption of cardiomyocyte architecture and functional impairment. By contrast, interstitial fibrosis stiffens the tissue, thereby

compromising cardiac performance (47). Similar to the findings of Injac *et al* (42), the present study showed a decrease in pathological changes in heart tissue in the FUL/C-60 group, suggesting that fullereneol C₆₀ exhibited cardioprotective effects and alleviated sepsis-induced cardiotoxicity. The antioxidant and anti-inflammatory actions of fullereneol C₆₀ may represent the underlying mechanisms of this protective effect.

Carbon-based antioxidants with neuroprotective and neuroregenerative properties may help limit the effects of sepsis on the brain (48,49). In parallel with our previous study, which showed the effects of fullereneol C₆₀ on kidney and lung tissues (29), the changes observed in the levels of TBARS and CAT enzymes, indicators of oxidative stress processes in brain tissue and histopathological findings also support the neuroprotective effect of fullereneol C₆₀. The elevated TBARS level observed in the brain tissue of the DM/SEP group indicated that oxidative stress triggered neurodegenerative processes. However, the reduction in TBARS levels in the FUL/C-60 group suggested that fullereneol C₆₀ attenuated free radical-induced damage. In addition, the increase in CAT enzyme activity indicated the potential of fullereneol C₆₀ to support antioxidant defense mechanisms in the brain.

Based on the findings of the present study, fullereneol C₆₀ may be considered a potential prophylactic agent for preventing sepsis-induced organ tissue damage in diabetic rats. Given its ability to suppress sepsis-induced oxidative stress and inflammation, thereby reducing multiple organ failure, further experimental research is needed to determine its therapeutic efficacy and safety, supporting the clinical application of fullereneol C₆₀.

However, the present study exhibits certain limitations. Markers of oxidative stress in the heart, systemic inflammatory markers (including cytokines) and the molecular mechanisms of action of fullereneol C₆₀ were not studied. In the present study, based on previous research, fullereneol C₆₀ was administered as a single dose. Samples were taken from rats 24 h after laparotomy, therefore the long-term effects of fullereneol C₆₀ were not studied. Further research is required to understand the effects and mechanisms of action on dose-dependent response. Another limitation of the present study is the absence of a non-diabetic sepsis (CLP-only) group. Inclusion of such a group would have allowed more precise differentiation between the effects of diabetes and sepsis on organ injury and oxidative stress parameters. Due to ethical restrictions on the maximum number of animals permitted, the present experimental design focused primarily on the diabetic sepsis model. Future studies incorporating both diabetic and non-diabetic sepsis groups are warranted to further clarify the interaction between diabetes and sepsis in the context of fullereneol C₆₀ treatment.

In conclusion, histopathologically, the present study found that fullereneol C₆₀ reduced oxidative stress and inflammation in a streptozotocin-induced diabetic rat sepsis model, thereby reducing sepsis-induced damage in liver, heart and brain tissues. The structural and biochemical improvements observed in liver, heart and brain tissues in the present study suggested that fullereneol C₆₀, with its antioxidant and anti-inflammatory properties, may be beneficial in the preventive treatment of multiple organ failure in sepsis. The precise molecular mechanisms underlying these protective effects of fullereneol C₆₀ were not directly investigated in the present

study. Future studies incorporating molecular analyses such as the evaluation of Nrf2/ARE pathway activation, NF-κB signaling modulation, mitochondrial function markers and apoptosis-related proteins will therefore contribute to an improved understanding of the safety and therapeutic efficacy of fullereneol C₆₀ and its organ-protective effects in diabetic sepsis, as well as provide a promising basis for new approaches to sepsis treatment in the diabetic population.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MAr, ÖK, KD, NŞ, LF and HB conceived and designed the study, and contributed to data analysis and interpretation. ÖK, HB and KD performed the experimental procedures. MK, FE, MAr and ŞŞ conducted the biochemical and histopathological analyses. ÖK and FE collected the samples. AB and IG contributed to data analysis and interpretation, and participated in the evaluation of the findings. AB, IG, KD, ÖK and MK confirm the authenticity of all the raw data. ÖK, MK, NŞ, LF, AB, IG, MAr, and ŞŞ critically revised the manuscript for important intellectual content. All authors have read and approved the final version of the manuscript, and agree to be accountable for all aspects of the work.

Ethics approval and consent to participate

Ethical approval for the present study was obtained from The Animal Research Committee of Gazi University (Ankara, Turkey; approval no. G.Ü.ET-24-046).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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