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# Carvacrol Attenuates Amikacin-Induced Nephrotoxicity in the Rats

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## ABSTRACT

**Objective:** Amikacin (AK) is a wide-spectrum antibiotic routinely used to treat gram-negative and some gram-positive bacterial infections. However, its use is limited due to its potential to cause nephrotoxicity due to an increase in reactive oxygen radicals. The main goal of this study was to investigate the effect of carvacrol (CAR) on AK-induced nephrotoxicity in rats.
**Methods:** Thirty-two Sprague Dawley rats were randomly separated into four groups: the control (0.9% NaCl solution and sunflower oil), AK (400 mg/kg), CAR+AK (80 mg/kg CAR+400 mg/kg AK), and AK+CAR (400 mg/kg AK+80 mg/kg CAR) groups. AK and CAR were administered intramuscularly and orally, respectively for 7 days. Blood and kidney tissue samples were collected at the end of the experiment. The level of catalase, superoxide dismutase, malondialdehyde, and reduced glutathione, which are parameters of oxidative stress, were detected while comparing renal function and histopathological changes.
**Results:** Histopathological findings (necrotic changes, dilatation and inflammatory cell infiltration) were significantly greater in the AK group than in the control group. Additionally, significant weight loss was detected in the rats in the AK group. CAR treatment, both before and after AK administration, significantly improved nephrotoxicity histopathologically ($p<.05$). However, the same improvement was not identified biochemically.
**Conclusion:** CAR treatment significantly improved nephrotoxicity both before and after AK administration, suggesting that carvacrol has a protective effect against AK-induced kidney damage at the histopathological level.
**Keywords:** Antioxidant, amikacin, carvacrol, nephrotoxicity, oxidative stress, rat

## Introduction

Acute kidney injury (AKI), known as acute renal failure, is a syndrome characterized by a rapid decrease in glomerular filtration (Liu et al., 2020). AKI has been reported in nearly 10-15% of all hospitalized patients, and the incidence of AKI is even greater (over 50%) among patients admitted to intensive care units (ICUs) (Ronco et al., 2019).

Aminoglycosides exhibit antibacterial effects by inhibiting bacterial protein synthesis via reversible binding to the 16S ribosomal RNA of the 30S ribosome with high affinity (Ahmed et al., 2020). Amikacin (AK) is an aminoglycoside-derived antibiotic. It is often used in intensive care units to treat life-threatening bacterial infections, especially those caused by gram-negative aerobes and gram-positive Staphylococcus aureus (Abdel-Gayoum et al., 2015, Polat et al., 2006). The essential advantages of AK are its high antibacterial activity, rapid effect, synergistic activity when combined with beta-lactam antibiotics, low-cost and low resistance (Kara et al., 2016). Despite its high efficacy, the clinical use of AK is restricted due to its ototoxicity and nephrotoxicity (Raeeszadeh et al., 2021). A well-documented side-effect of AK is nephrotoxicity, which is characterized by tubular necrosis, particularly in experimental animal studies and clinical observations in humans (Sweileh 2009, Parlakpinar et al., 2003). Most aminoglycosides are excreted in the urine without being metabolized. Some AKs also accumulate selectively in the renal cortex (Selim et al., 2017). Aminoglycosides, including amikacin, are picked up by renal proximal tubular cells through endocytosis, and their accumulation can damage various cellular structures. (McWilliam et al., 2017, Polat et al., 2006).

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Various nephrotoxicity mechanisms, including kidney tubular toxicity, glomerular injury, inflammation, crystal nephropathy, and thrombotic microangiopathy, have been explored (Al-Kuraishy et al., 2019, Ozer et al., 2020, Kang et al., 2011). Substantial evidence supports the role of reactive oxygen species (ROS) in AK-induced nephrotoxicity, where their accumulation leads to oxidative stress and disrupts the antioxidant/oxidant balance. AK induces nephrotoxicity characterized by oxidative stress through elevating the levels of malondialdehyde (MDA) and decreasing the levels of glutathione (GSH). Despite these known nephrotoxicity mechanisms, the renal toxicity of AK is not fully known. Additionally, various experimental studies have shown that nephrotoxicity can be prevented by several antioxidants (Parlakpinar et al., 2004, Parlakpinar et al., 2003, Parlakpinar et al., 2006, Abdelhamid et al., 2020).

In recent years, various studies have been conducted to identify effective renoprotective compounds that could be beneficial in clinical practice (Rehman et al., 2014). CAR is indeed a monoterpene phenol found in the essential oils of different Labiatae plants, such as Satureja, Origanum, Corydothymus, Thymus, and Thymbra (Melo et al., 2011, Ili and Keskin 2013). It is widely used as an additive in the food industry (Ultee et al., 1999). CAR is known for its biological and pharmacological activities, such as anticancer, antioxidant, anti-inflammatory, antibacterial, antifungal, and hepatoprotective activities both in vitro and in vivo (Potočnjak and Domitrović 2016, Suntres et al., 2015, Ili and Keskin 2013, Ghorani et al., 2021). CAR has been shown to have anti-inflammatory effects by increasing the synthesis of IL-10 and lowering the production of proinflammatory mediators such as IL-1β (da Silva Lima et al., 2013). The major biological and pharmacological activities of CAR are shown in Figure 1.

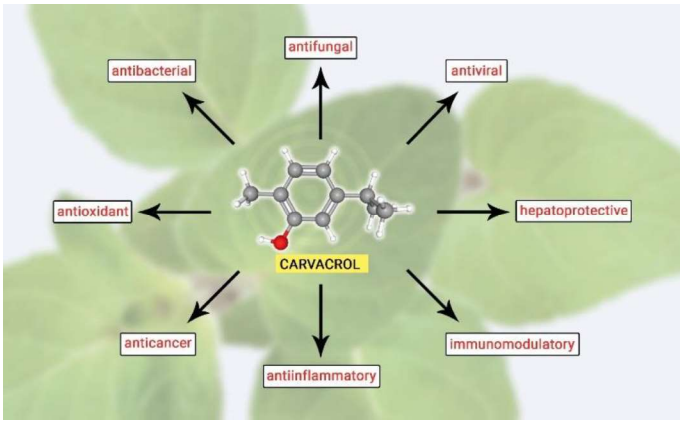


**Figure 1.** CAR's major biological and pharmacological activities.

Some studies have investigated the effects of CAR on nephrotoxicity; however, there are no studies on the protective and therapeutic effects of CAR, a known potent antioxidant, on AK-induced nephrotoxicity. The hypothesis of this study is that CAR, whose antioxidant and anti-inflammatory effects have been shown in previous studies, may be effective in treating nephrotoxicity, in which reactive oxygen species and inflammation play role. Therefore, this study was designed to investigate the possible antioxidant and anti-inflammatory effects of CAR on AK-induced nephrotoxicity in rats.

## Methods

### Animals

For the present study, 32 female 4- to 6-month-old Sprague–Dawley rats with weights of 218–320 g were obtained from the Inonu University Laboratory Animals Research Center and maintained in a controlled room with a controlled temperature (21 ± 2 °C) and humidity (60 ± 5%) and a 12:12 h light: dark cycle. Rats were fed with a standard chow pellet diet and provided with tap water ad libitum. Animals were randomly assigned to different experimental groups to assemble and process the data and to allow investigators to blindly analyze the treatment groups. Almost all of the experiments in the study were performed according to the National Institutes of Health Animal Research Guidelines and ARRIVE guidelines (Çolak % Parlakpınar, 2012). The protocol of this study was authorized by the Committee of Ethics on Animal Research (reference no: 2015/A-79) of the Faculty of Medicine, Inonu University, Malatya, Türkiye. A simple randomization technique was applied to allocate the rats to different experimental groups to minimize bias and enhance the credibility of the findings in the experiment.

### Chemicals

AK (Amikozit 500 mg®, Eczacibasi Corp., İstanbul, Türkiye), CAR (CAS number: 499-75-2, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and ethyl carbamate (urethane® CAS number: 51-79-6, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were purchased.

### Experimental design

As described in Figure 2, thirty-two female Sprague Dawley rats were randomly assigned to four groups (n=8 for each group) as listed below:

1. Control group: Rats were given 0.5 mL of 0.9% NaCl solution via intramuscular (i.m.) administration and 1 mL

of sunflower oil via a per-oral (p.o.) administration of one dose daily for 7 days.

2. AK group: Rats were given 0.5 mL of AK (400 mg/kg) via the i.m. route and 1 mL of sunflower oil via p.o. one dose daily for 7 days.

3. CAR+AK group: CAR (80 mg/kg) was applied via the p.o. route. One dose daily for 7 days before the first dose of AK (400 mg/kg) was administered via the i.m. route, and one dose daily and continued for 7 days to assess the protective effects of CAR against AK-induced nephrotoxicity.

4. AK+CAR group: After one dose of AK (400 mg/kg) daily for 7 days, one dose of CAR (80 mg/kg) was given via the p.o. route daily for 7 days.

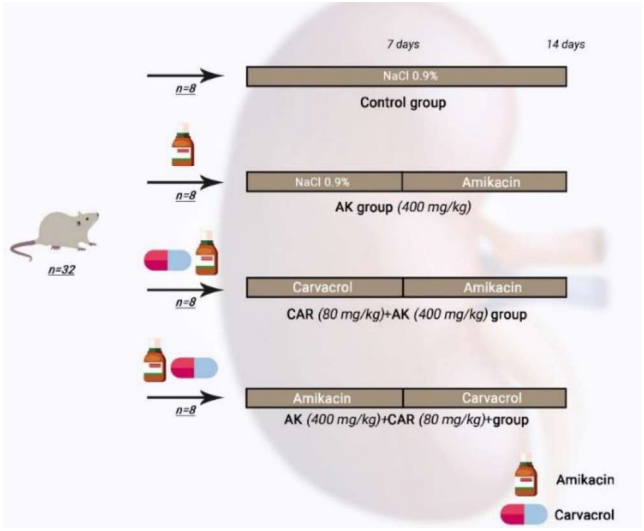


**Figure 2.** Schematic representation of the experimental design.

Each rat was weighted and then anesthetized via intraperitoneal (i.p.) administration of ethyl carbamate at a dose of 1.2 g/kg 24 h after the last injection. Then, blood samples and kidney tissues were obtained from anesthetized rats, and histopathological and biochemical analyses were performed. Kidneys were quickly extracted, decapsulated, and split equally into two longitudinal segments. Half of the tissue was fixed with formalin for histopathological analysis, and the remaining half of the kidney tissue was stored at -70 °C for biochemical analysis. Blood samples were obtained in tubes without anticoagulant to determine blood urea nitrogen (BUN) and creatinine (Cr) levels. Histopathological examinations (tubular dilatation, infiltration of inflammatory cells, and necrosis under a light microscope) and biochemical evaluations [catalase (CAT), superoxide dismutase (SOD), MDA, and reduced GSH] were performed at the end of the study protocol.

**Biochemical analysis**

After centrifuging blood samples from the rats at 3500 rpm for 10 min, the serum samples were separated in Eppendorf tubes and stored at -80 °C. One day before the biochemical analysis, the frozen samples were transferred to the +4 °C unit for thawing. Afterwards, serum parameters such as BUN and Cr were studied at Inonu University Turgut Ozal Medical Center Laboratories (Abbott Architect c16000).

The levels of CAT, MDA, SOD, and GSH in the renal tissue were measured. Protein determination in the tissue was performed by Biuret protein analysis using bovine serum albumin as a standard (Hiller et al., 1948).

The activity of MDA (an important marker of lipid peroxidation) was calculated according to Uchiyama and Mihara (Uchiyama & Mihara 1978). The kidney samples were homogenized on ice for 1 min at 15,000 rpm in a 1.15% KCl solution to form a 10% homogenate. This homogenate was directly used for analyzing MDA. The prepared solutions were added to the test tubes and vortexed, and the tubes were left in boiling water at 95 °C for 1 h. The tubes were vortexed for 5 min after adding two ml of n-butanol and then centrifuged at 3,000×g for a minute. The absorbances were measured by using a spectrophotometer at 532 nm. The value of lipid peroxides was computed as the TBARS of lipid peroxidation, and the results are presented in nmol/g tissue according to the adjusted standard graph.

The detection of GSH was performed according to the methods of Ellman (Ellman 1959). Kidney tissue samples were homogenized on ice for 1-2 min at 15,000 rpm to form a 10% homogenate, and the homogenate tissues were subsequently centrifuged at 3,000 rpm for 15 min at +4 °C. To prepare the samples for GSH analysis, trichloroacetic acid (TCA) solution was added to the resulting supernatant, which was mixed and centrifuged again. The processed solutions were placed in test tubes and vortexed, and the color intensity was measured at 410 nm in a spectrophotometer after 5 min. The results were evaluated from the standard chart of GSH and are presented as nmol/g wet tissue.

The activity of tissue SOD was calculated according to the methods of Sun et al (Sun et al., 1988). Kidney tissue samples were homogenized on ice for 1 min at 15,000 rpm to form a 10% homogenate, which was subsequently centrifuged at 10,000 rpm for 20 min. A prepared mixture of chloroform/ethanol at a ratio of 3 to 5 was added to

the supernatant. Then, the sample was centrifuged at 5,000 rpm for 20 min at +4 °C. Afterwards, for CuZn-SOD analysis, the top clear white chloroform phase was carefully pipetted and used. The arranged test tubes were centrifuged for 20 min at 25 °C. At the end of the process, CuCl2 was added to each tube, and the reaction was stopped (0.8 mmol/L). A spectrophotometric evaluation was performed at 560 nm. The absorbance of both the blank and the samples was recorded, and the enzyme activity was measured. SOD enzyme activity was given as U/g protein.

The activity of tissue CAT was measured based on the method of Luck (Luck 1974). Kidney tissue samples were homogenized on ice for 1 min at 15,000 rpm to form a 10% homogenate. Then, the same homogenate tissues were centrifuged at 10,000 rpm for 20 min, and CAT analysis supernatant was used. The absorbance of the spectrophotometer was adjusted to zero by a blunt and was brought to 240 nm. After the supernatant was added to the sample tubes, the absorbance at 240 nm was measured. Then, the absorbance decreased gradually, followed by repeated readings every 15th sec for 90 sec. At the end of the period, the absorbance value was recorded. The time interval of the linear absorbance reduction was evaluated. CAT activity is presented as K/g protein.

### Histopathological analysis

Finally, renal tissue samples were fixed in 10% formaldehyde and embedded in paraffin. After tissue follow-up procedures, four- to five-µm-thick slices of paraffin blocks were cut, prepared and subjected to hematoxylin-eosin (H&E) staining for general histological evaluation. Kidney tissues were analyzed for tubular dilatation, inflammatory cell infiltration, and necrosis. For semiquantitative scoring of each variable, the following scale was used: 0, normal tissue; 1, <25% of the entire area was damaged; 2, 25–50% of the entire area was injured; and 3, >50% of the entire area was damaged. A Leica Q Win Image Analysis System (Leica Micro Imaging Solutions Ltd., Cambridge, UK) with a Leica DFC-280 research microscope was used to carry out histopathological analysis.

### Data analysis

The required power and sample sizes used in the experiment were defined by using statistical power analysis to detect even minor effects. Power analysis using the type I error probability to detect bidirectional variance between experimental groups (α=0.05) and type II error probability (β=0.20), along with past laboratory results, showed that the minimum sample size required to detect a significant difference in the levels of Cr in the pilot study should be at least 8 per group (24 in total) according to the Web-Based Sample Size and Power Analysis Software required for each experimental group (Arslan et al., 2018). The Kolmogorov–Smirnov test was used to verify the normality of the distribution. Based on the normality tests, the measurable variabilities of all groups in the study did not show a normal distribution. Consequently, for the statistical assessment of histopathological results, Kruskal–Wallis variance analysis was used. For comparisons between paired groups, the Mann–Whitney U test was used. A p value less than 0.05 was considered to indicate statistical significance. IBM SPSS Corp., Armonk, NY for Windows package program was used for the data analysis. The data are presented as the median (minimum-maximum).

## Results

### Experimental toxicity and body weight

During the experiment, none of the animals died due to interventional procedures or any other cause. AK application led to significant weight loss compared to that in the control group ($p<.05$). CAR treatment before and after AK administration did not significantly improve body weight. Rat weight and serum and tissue biochemical parameters are presented in Table 1.

### Biochemical findings

### Kidney function tests

AK application caused a significant increase in the BUN level in all groups compared to that in the control group ($p<.05$). However, CAR treatment before and after AK administration did not significantly increase the BUN level

### Tissue biochemical findings

The application of AK did not significantly change the level of MDA compared to that in the control group. Additionally, CAR administration before and after AK treatment resulted in a statistically significant decrease in MDA levels (P>0.05). The application of AK caused a significant increase in the GSH level compared to that in the control group. However, CAR administration before and after AK treatment did not cause significant changes in GSH levels (P>0.05). Administration of AK did not cause any significant changes in the level of SOD compared with

that in the control group. However, CAR administration both before and after AK treatment resulted in a statistically significant increase in the level of SOD compared to that in the control group. Administration of AK caused an insignificant decrease in the level of CAT compared to that in the control group. There was also a significant increase in the level of CAT in the AK-CAR group compared to that in the AK group.

**Table 1.** Rat weight, serum and tissue biochemical parameters.

| Parameters* | Groups* | | | | *p* |
|---|---|---|---|---|---|
| | Control | AK | CAR+AK | AK+CAR | |
| Rat weight (g) | 279[a,b] (254-307) | 241 (235-320) | 251 (218-272) | 262 (237-284) | .0117 |
| BUN (mg/dL) | 24.57[a,b,c] (21.35-26) | 31.27 (20.37-74.31) | 33.7 (25.21-95.77) | 34.87 (31.47-80.37) | .0027 |
| Creatinine (mg/dL) | 0.6[c] (0.58-0.68) | 0.66[c] (0.5-1.38) | 0.72[c] (0.57-1.88) | 0.9 (0.82-1.53) | .0034 |
| MDA (nmol/g wet tissue) | 175.78[a,b,c] (154.36-204.68) | 149.6 (107.44-190.4) | 115.6 (104.04-169.32) | 129.54 (114.92-162.52) | .0097 |
| GSH (nmol/g wet tissue) | 589.13[a,b,c] (488.4-779.4) | 855.72 (563.7-1056.16) | 828.25 (543.35-954.41) | 768.21 (667.48-1058.2) | .0198 |
| SOD (U/g protein) | 573.99[b,c] (511.67-708.48) | 637.33[b] (224.14-851.39) | 726.38 (679.93-822.68) | 701.95 (585.85-808.13) | .0092 |
| CAT (K/g protein) | 2479.18[c] (1667.6-5012.1) | 2467.8[c] (1294.4-3930.81) | 2404.12 (1333.41-4259.17) | 3912.29 (2614.4-4331.32) | .045 |

[a]Significant compared to AK group ($p$<.05).
[b]Significant compared to CAR+AK group ( $p$<.05).
[c]Significant compared to AK+CAR group ( $p$<.05).
*Data are expressed as median (min-max).
MDA, malondialdehyde; GSH, reduced glutathione (GSH); SOD, superoxide dismutase; CAT, catalase;

### Histopathological findings

Kidney tissues in the control group exhibited a normal histological structure (Figure 3A). Nonetheless, necrotic changes and dilatation were detected in the tubules in the cortical area of the AK group (Figure 3B). Infiltration of inflammatory cells in the interstitial tissue was another important finding detected in the AK group (Figure 3C). In terms of these changes, the difference between the AK and control groups was statistically significant ($p$<.05). CAR administration before AK significantly reduced all histopathological changes observed in the AK group ($p$<.05) (Figure 3D and 3E). Tubular dilatation and necrosis were reduced considerably in the CAR group after AK ($p$<.05), while infiltration remained similar to that in the AK group (Figure 3F and 3G). Moreover, the administration of CAR before and after AK treatment did not cause any changes in tubular dilatation or inflammatory cell infiltration. However, tubular necrosis was significantly less common in the CAR group before AK surgery ($p$<.05). The histopathological evaluation results of the groups are given in Table 2.

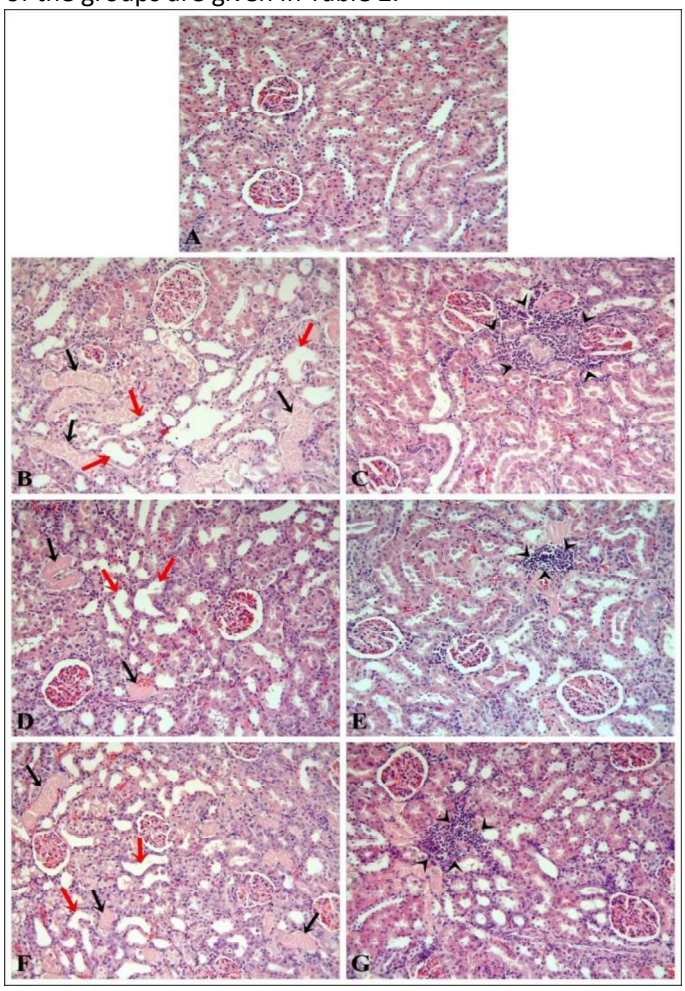


**Figure 3.** The renal cortical tissue has a normal histological appearance in the control group (A). Tubular necrosis, dilatation and infiltration in the interstitial space are seen in the AK group (B and C). There was a significant improvement in histopathological changes in the CAR+AK group (D and E). Tubular changes decreased significantly in the AK+CAR (F and G) group, but infiltration continued similar to the AK group. Black arrows indicate tubular necrosis, red arrows indicate tubular dilatation, arrowheads indicate infiltration. H-E; x20.

### Discussion

The goal of this study was to reveal the protective and therapeutic effects of CAR treatment on oxidative casualties and disruption of the antioxidant defense system via biochemical and histopathological analysis. According to our results, histopathological changes (necrotic changes, dilatation and inflammatory cell infiltration) were significantly greater in the AK group than in the control group. All nephrotoxicity findings were significantly reduced by the administration of CAR before AK in the AK group. However, tubular dilatation and

necrosis were reduced significantly in the CAR group after AK treatment, whereas infiltration was not changed. Tubular necrosis was significantly lower in the CAR+AK group than in the AK group.

Oxidant products, such as reactive oxygen species (ROS) and nitrogen species are physiologically released as a result of cellular activities but remain in a physiological balance (Baltaci et al., 2019). Cellular antioxidant mechanisms provide this balance (Gunata & Parlakpinar, 2020). However, a shift in this physiological balance in favor of oxidants due to endogenous or exogenous causes plays a role in the pathogenesis of many diseases, such as diabetes mellitus, atherosclerosis, myocardial infarction, renal failure, rheumatoid arthritis, and nephrotoxicity (Gunata & Parlakpinar, 2020, Abdel-Daim et al., 2019, Kapucu 2021). ROS damage many structures, such as the cell membrane and nucleus (Gunata & Parlakpinar, 2020, Zare Mehrjerdi et al., 2020).

**Table 2.** Histopathological evaluation results

| Groups | Parameters* | | |
|---|---|---|---|
| | Tubular dilatation | Tubular necrosis | Inflammatory cell infiltration |
| **Control** | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) |
| **AK** | 1.0 (0.0-3.0)[a] | 1.0 (0.0-2.0)[a] | 1.0 (0.0-0.3)[a] |
| **CAR+AK** | 0.0 (0.0-2.0)[b] | 0.0 (0.0-1.0)[b,c] | 0.0 (0.0-2.0)[b] |
| **AK+CAR** | 0.0 (0.0-2.0)[b] | 0.0 (0.0-2.0)[b] | 0.0 (0.0-2.0) |

[a] Significant increase compared to control group ($p<.05$).
[b] Significant decrease compared to the AK group ($p<.05$).
[c] Significant decrease compared to AK+CAR group ($p<.05$).
*Data are expressed as median (min-max).

Nephrotoxicity is a significant clinical concern associated with the use of aminoglycoside antibiotics, which are widely used to treat gram-negative infectious diseases (Parlakpinar et al., 2006). The kidney is an organ that maintains homeostasis and removes metabolic products from the body. Kidney functions include maintaining water and electrolyte balance, producing various hormones, removing bioactive substances that affect body functions, regulating blood pressure, synthesizing erythropoietin, regulating vitamin D production, and regulating calcium metabolism (Sahay et al., 2012). Approximately 25% of cardiac output passes through the kidneys. Therefore, drugs can damage the kidneys and have potentially toxic effects (Peasley et al., 2021). Medications are indeed a relatively common cause of AKI. Drug-induced nephrotoxicity is a significant contributor to AKI in both adult and pediatric populations. Prospective cohort studies have reported that approximately 14- 26% of AKI cases in adults and 16% of AKI cases in hospitalized children are due to drug-induced nephrotoxicity. Drug-induced nephrotoxicity is more common in hospitalized patients, especially patients in intensive care units (Perazella 2018, Hoste et al., 2015). It is predicted that approximately 25% of patients receiving aminoglycoside therapy may develop nephrotoxicity (Lopez-Novoa et al., 2011). Various risk factors that facilitate the development of aminoglycoside-associated nephrotoxicity have been identified. These risk factors include patient-specific factors such as advanced age, impaired renal function, dehydration, hepatic dysfunction, hypothyroidism, metabolic acidosis, and sodium depletion. In addition, long treatment duration, higher doses of the drug, the use of divided doses of the drug, and the use of various drugs that are eliminated via the renal route are also important risk factors (Wargo and Edwards 2014).

Aminoglycosides show concentration dependent, bactericidal activity (Mirazi et al., 2021). Due to the widespread use of aminoglycosides, side effects such as ototoxicity and nephrotoxicity have become more pronounced. Despite its side effects, it continues to be widely used due to its various advantages. The essential advantages of AK are its high antibacterial activity, rapid effect, low resistance, synergistic activity with beta-lactam antibiotics, and low cost (Kara et al., 2016). AK is a broad-spectrum aminoglycoside derivative drug that causes nephrotoxicity via many mechanisms. These mechanisms include decreased blood supply to the renal tissue, decreased glomerular filtration rate, and tubular cytotoxicity. The contraction of mesangial cells and the release of vasoconstrictor hormones such as angiotensin 2 may cause a decrease in the blood supply of renal tissue (Krause et al., 2016, Abdel-Daim et al., 2019).

In the present study, AK led to significant renal dysfunction, as demonstrated by the substantial increase in the serum urea concentration. Nephrotoxicity caused by aminoglycoside is an important and common cause of morbidity, especially in hospitalized patients. This causes a significant additional treatment cost (Bulut et al., 2016). Most aminoglycosides are eliminated by the renal route, usually without being metabolized. Some drugs accumulate in the proximal segments of renal tubules, and the drug concentration in the proximal segments of renal tubules is greater than that in plasma (Bulut et al., 2016). This accumulation is associated with nephrotoxic effects. The accumulation of AK increases oxidative stress by generating free radicals (Bulut et al., 2016). Oxidative stress caused by AK leads to cellular dysfunction and DNA damage (Xiong et al., 2015). Oxygen radicals are thought to play important roles in the pathogenesis of AK-induced nephrotoxicity (Yang et al., 2017). Therefore, antioxidants

are valuable for controlling AK-induced nephrotoxicity. It is also known that TNF and Nrf-2 expression in renal tissue is increased in AK-induced nephrotoxicity (Selim et al., 2017, Abd El-Kader and Taha 2020, El-Kashef et al., 2015).

Histopathological studies have shown that tubular necrosis is the main cause of nephrotoxicity (Asci et al., 2015, Parlakpinar et al., 2004). Glomerulus obstruction, proinflammatory cell migration, tubule dilatation, bleeding, and tubule degeneration were observed in the AK-induced nephrotoxicity group (Asci et al., 2015). This may be due to the generation of highly reactive radicals due to oxidative stress caused by AK. CAR administration before AK significantly decreased all histopathological changes observed in the AK group (Figure 3D and 3E). Tubular dilatation and necrosis were reduced considerably in the CAR group after AK surgery (Figure 3F and 3G). Tubular necrosis was significantly less common in the CAR group before AK resection. These findings show that CAR-T-cell therapy has both histopathalogically protective and therapeutic effects against oxidative kidney damage.

Although other studies have shown that AK decreases the activities of CAT and SOD enzymes in kidney tissue, we did not observe any significant changes in the levels of these antioxidant enzymes in our study protocol (Abdel-Daim et al., 2019). In accordance with our study results, other studies have shown that the levels of antioxidant enzymes such as CAT and SOD may not change due to increased oxidation and antioxidant usage (Yılmaz et al., 2018, Ohta and Nishida 2003). In another study, Cu(II)-aminoglycoside complexes were shown to be formed by holding the copper ions of aminoglycosides (Szczepanik et al., 2004). Ulusoy et al., showed that MDA, total oxidative status, and oxidative stress indices increased in an experimental AK-induced nephrotoxicity model (Ulusoy et al., 2012, Ahmed et al., 2021). Similarly, Kose et al., reported that MDA levels increased significantly in an AK-induced nephrotoxicity model in rats (Kose et al., 2012). However, no significant increase in the level of MDA was detected between the AK-induced nephrotoxicity group and the control group in our study. In contrast to the expected increase in MDA levels under conditions of oxidative stress or oxidant exposure, some studies have reported no change or even a decrease in MDA levels in certain contexts (Garcia et al., 2020, Lima et al., 2019, Ubani-Rex et al., 2017). Importantly, not all lipid peroxidation processes result in the production of MDA. Additionally, MDA can be formed through reactions other than lipid peroxidation (Jenkins 2000, Sharma et al., 2021). Animal and human studies have shown that MDA levels remain unchanged despite the expectation that increased oxidation would lead to elevated levels of MDA, a marker of lipid peroxidation (Ma'rifah et al., 2019, McGrath et al., 2001, Kamendulis et al., 1999). A significant increase in the level of BUN was detected by comparing outcomes between the AK-induced nephrotoxicity group and the control group in the AK-induced nephrotoxicity model (Abdel-Daim et al., 2019, Parlakpinar et al., 2006). Similarly, in the present study, we showed that the BUN level was greater in the AK-induced nephrotoxicity group than in the control group. The fact that the BUN and Cr parameters were studied without using a rat specific kit can be considered among the limitations of this study.

CAR is a monoterpene phenol found in the essential oils of Labiatae, such as Satureja, Origanum, Coridothymus, Thymus, and Thymbra (Ili & Keskin, 2013, Shahrokhi Raeini et al., 2020). CAR has lipophilic properties and various biological and pharmacological activities, including anticancer, antioxidant, antibacterial, antifungal, and hepatoprotective activities both in vitro and in vivo (Figure 1) (Suntres et al., 2015, Shahrokhi Raeini et al., 2020, Oliveira et al., 2012). It has been suggested as a natural food preservative for the food industry, mainly due to its safety, flavoring, quality and antimicrobial activities (Mishra et al., 2018).

Recent studies in rats revealed that CAR treatment has beneficial effects on ameliorating oxidative stress-induced damage in multiple organs, such as the brain, liver, and kidneys (Samarghandian et al., 2016). The anti-inflammatory and antioxidant properties of CAR have been demonstrated in experimental models of various inflammatory conditions, including arthritis, colitis, asthma, ischemia/reperfusion injury, and sepsis (Banji et al., 2014, Khosravi and Erle 2016, Arigesavan and Sudhandiran 2015, Suo et al., 2014). A study of experimental renal ischemia/reperfusion injury in rats showed that CAR treatment has beneficial effects on tubular atrophy, dilatation, brush border loss, and hydropic epithelial cell degeneration (Ozturk et al., 2018).

## Conclusion

We investigated for the first time the effects of CAR in this experimental AK-induced nephrotoxicity model in rats. According to the results of the present study, CAR administration significantly improved histopathological injury in AK-induced nephrotoxicity rats. Additionally, the consumption of CAR improved the BUN concentration in the AK-induced nephrotoxicity group. However, CAR did

not considerably improve biochemical markers in kidney tissue. However, we share some of our biochemical results, which we cannot fully explain, to shed light on future studies. Furthermore, we recommend further research to determine the clinical applicability and effectiveness of the CAR. Plant extracts standardized by further analysis of medicinal plants containing CARs can be used in complementary treatments in the clinic.

**Ethics Committee Approval:** This study protocol was approved by the Ethics Committee on Animal Research (reference no: 2015/A-79) of the Faculty of Medicine, Inonu University, Malatya, Türkiye.
**Author Contributions:** Concept - H.P., Y.A.; Design- Y.F.C, A.P., H.P.; Supervision- H.P., Y.A.; Resources- Y.A., H.P.; Data Collection and/or Processing- O.O., I.K., N.V., K.T., A.K.A.; Analysis and/or Interpretation- O.O., I.K., N.V., K.T., A.K.A.; Literature Search- Y.A.; Writing Manuscript- Y.A.; Critical Review- H.P., Y.A.
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# Prophylactic Effects of Parsley (Petroselinum crispum), on Sepsis Model Via Cecal Ligation and Puncture Procedure

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**ABSTRACT**

**Objective:** Sepsis causes the release of free oxygen radicals that disrupt membrane integrity, and damage to Mitochondria due to the production of free oxygen radicals and oxidation leads to exacerbation of sepsis, Cecal ligation and puncture (CLP) in rats mimics the characteristics and course of clinical sepsis (Hubbard et al., 2005).

**Methods:** We evaluated the antioxidant effects of Petroselinum crispum (Pc) in a cecal ligation and puncture (CLP)-induced rat sepsis model, in a rat model of sepsis caused by cecal ligation and puncture (CLP). *Wistar albino* rats were separated into four groups of eight groups: a sham group with incised and sutured abdomens; a Pc extract (PcE) group, which was given 2 g/kg parsley extract for 14 days by gastric gavage; a CLP group, which was subjected to sepsis caused by CLP; and a PcE + CLP group, which was given parsley extract for 14 days. PcE was given for 14 days, after which sepsis was induced via the CLP procedure. The groups were compared in terms of hemogram, biochemical and histological characteristics.

**Results:** The administration of PcE before CLP-induced sepsis increases neutrophil counts, PLTs and TASs, which decrease with sepsis, and decreases biochemical changes (BUN, AST, ALT, LDH, TOS, and OSI), which increase with sepsis, to protect against sepsis. Compared with that in the CLP group, the severity of intestinal infiltration was significantly lower in the PcE + CLP group; however, the degree of epithelial damage in the PcE + CLP group was similar to that in the CLP group. In the PcE + CLP group, the crypt and villus lengths were greater, and the decrease in Paneth cell degranulation intensity was greater than that in the CLP group.

**Conclusion:** Additionally, the morphology of the cells in the PcE + CLP group was similar to that in the sham group. PcE has potential as a prophylactic agent for sepsis.

**Keywords:** Oxidative stress; parsley; Petroselinum crispum; rat; sepsis

## Introduction

Sepsis is an irregular and excessive systemic inflammatory response to an infection. Despite improvements in treatments, it has a high mortality rate and requires early intervention (Neviere et al., 2017). The mortality rate for sepsis is approximately 30%; mortality increases as it progresses from severe sepsis to septic shock and multiple organ failure syndromes (Martin et al., 2003). One factor contributing to the pathogenesis of sepsis is excess production of free oxygen radicals. Mitochondria are most susceptible to damage by oxidation. The release of free oxygen radicals eliminates the membrane potential and disrupts membrane integrity (Bone, 1991; Cohen, 2002; Zhou et al., 2015).

Cecal ligation and puncture (CLP) in rats mimics the characteristics and course of clinical sepsis (Hubbard et al., 2005). The CLP model can be used to vary the severity of sepsis by controlling the size of the perforation (Walley et al., 1996).

Petroselinum crispum, also known as parsley, can be grown almost anywhere and has been used traditionally for the treatment of many maladies. Apigenin, cosmosiin oxypeucedanin hydrate, myristicin, apiol, cnidil and apiin have been detected in aqueous extracts of Petroselinum crispum leaves.

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Additionally, coumarins, carotenoids and other compounds are present in various parts of parsley plants (Farzaei et al., 2013). Some compounds in parsley exhibit antioxidant properties (Wong and Kitts, 2006).

We investigated the protective effects of Petroselinum crispum extract on preventing the formation of free oxygen radicals and oxidative stress due to sepsis.

## Methods

### Animals

The Ethics Committee for Experimental Animal Research approved our study (Reference no. 2015/A-34). Our study was performed according to the principles of the Animal Research Guidelines of the International Health Board in the Laboratory of Experimental Animal Production (EAP) ( https://www.ncbi.nlm.nih.gov/books/NBK54044/) with the permission of the Ethics Committee of Experimental Animals (We used 32 180–220 g female *Wistar albino* rats obtained from the EAP Center. The rats were housed at 21 ± 2 °C and 60 ± 5% humidity with a 12 h light:12 h dark cycle. Before the experiment, the rats were provided standard feed and water ad libitum. Experimental animals that did not develop sepsis with cecal ligation were excluded from the study.

### Cecal ligation and puncture (CLP)

The sepsis model was created via CLP. A 1 cm midline incision was made in the abdominal wall under anesthesia.

The cecum was exposed and ligated with a 4–0 silk suture and then punctured twice with a 22-gauge needle. A small amount of fecal content was released by gently squeezing the cecum and then repositioning it with a 4–0 silk suture. After the procedure, 1 ml of saline solution (SS) was administered subcutaneously to the animals in each group. After sepsis symptoms, including lethargy, fever, piloerection and diarrhea, were observed after 24 h, the animals were euthanized by bleeding after a 5 ml blood sample was collected from the inferior vena cava, and the experiment was terminated (Toscano et al., 2011).

### Experimental design

The rats were allocated to our groups of eight. 1) Sham-operated group. 2) Petroselinum crispum extract (PcE) group: 2 g/kg PcE was administered once daily for 14 days by orogastric gavage. 3) CLP group. 4) In the PcE + CLP group, after the application of 2 g/kg PcE by gavage once daily for 14 days, the rats were septic after CLP.

### Tissue samples

On day 15, under anesthesia with 100 mg/kg ketamine

**Table 1.** Comparison of the Serum Hemogram and Biochemistry Parameters among the Study Groups

| Parameters | Groups | | | | |
|---|---|---|---|---|---|
| | Sham | PcE | CLP | PcE+CLP | *p* value |
| **WBC (10^9/µL)** | 5.69 (2.31-9.92) | 6.35 (3.71-14.04) | 4.09 (3.26-9.76) | 4.36 (2.36-10.03) | .4073 |
| **HGB (g/dL)** | 15.9 (13.9-18.4) | 15.1[b] (14.1-16) | 18.7[c] (14.7-20.9) | 14.55 (13.3-18.2) | **.0301** |
| **LY (%)** | 64[b.c] (35.4-71.9) | 64.7[b.c] (31.8-85.2) | 21.3 (14.7-40.3) | 20.8 (10-41.7) | **.0002** |
| **MO (%)** | 4.6[b] (3.5-8.9) | 3.4[b.c] (2.6-23.2) | 9.4 (5.5-46.2) | 18.05 (2.8-32) | **.015** |
| **NE (%)** | 31.2[b.c] (24.6-55.3) | 31.6[b.c] (11.8-50.1) | 49.9 (31.4-75.5) | 54.55 (42.4-77.6) | **.003** |
| **EO (%)** | 0.2 (0-0.3) | 0.1 (0-7.7) | 0 (0-1.3) | 0.1 (0-5.7) | .5803 |
| **BA (%)** | 0.2 (0-0.3) | 0.2 (0-0.4) | 0.3 (0-0.5) | 0.2 (0-0.3) | .6258 |
| **PLT (10^9/µL)** | 683 (2,4-910) | 764 (675-875) | 623 (418-1395) | 647 (51-856) | .3103 |
| **Glucose (mg/dL)** | 114 (49-170) | 78 (62-132) | 32 (12-151) | 62.5 (32-114) | .1485 |
| **BUN (mg/dL)** | 18.63(12.06-4.17) | 14.82[b.c](12.75-16.04) | 67.32(12.89-22.09) | 49.65(12.94-08.52) | **.0307** |
| **Creatinine (mg/dL)** | 0.54[a] (0.47-0.63) | 0.45[b.c] (0.42-0.51) | 0.69 (0.49-1.24) | 0.54 (0.45-0.92) | **.0013** |
| **AST (U/L)** | 23.8[b] (18.9-65.3) | 44.3 (26.9-71.8) | 70.6 (22.0-177.3) | 47.1 (16.7-72.3) | **.0388** |
| **ALT (U/L)** | 40[b] (27-82) | 51[b] (39-83) | 197 (28-1060) | 127 (24-287) | **.0339** |
| **LDH (U/L)** | 186.8[b] (171.0-258.8) | 190.2[b] (110.8-274.6) | 332.5 (166.4-332.5) | 247.9 (147.9-332.5) | **.0213** |

Results are expressed as median (min–max). n= 8.

a: *p*<.05 versus PcE group. b: *p*<.05 versus CLP group . c: *p*<.05 versus PcE+CLP group.

**Table 2.** Comparison of the tissue TAS, TOS and OSI among the study groups in terms of intestinal tissue

| Parameters | Groups | | | | |
|---|---|---|---|---|---|
| | Sham | PcE | CLP | PcE+CLP | *p* value |
| **TAS (Trolox Eq/L)** | 0.94 (0.79-1.08) | 0.95 (0.78-1.12) | 0.91 (0.83-1.14) | 1.05 (0.95-1.17) | .0654 |
| **TOS (µmolH2O2 Eqv/L)** | 5.43 (4.3-5.85) | 8.46 (5.35-12.61) | 8.46 (5.35-10.5) | 8.31 (5-11.98) | .0575 |
| **OSI (Arbitrary unit)** | 5.52 [a] (4.36-6.45) | 8.19 (4.96-10.98) | 8.89[b] (6.06-5.01) | 7.38 (4.29-12.55) | .0405 |

The results are expressed as the median (min–max), n= 8.
a: *p*<.05 versus the CLP group, b: *p*<.05 versus the PcE+CLP group

and 10 mg/kg xylazine, the skin was shaved and sterilized with iodine, and laparotomy was performed. The rats were sacrificed by extracting 5 ml of blood from the inferior vena cava 24 h after CLP. The characteristics measured included a hemogram, LDH, AST, ALT, BUN, creatinine, glucose, bicarbonate, CRP and ASO. A 10 cm portion of intestinal tissue from the ascending colon, including the cecum, was placed in a 10% formaldehyde or histopathological examination; the remaining portion was frozen in liquid nitrogen and stored in a -35 °C freezer until biochemical measurements were performed. Two hundred milligrams of frozen tissue samples cut into pieces on dry ice were homogenized in PBS buffer (1:9, w/v) via a manual glass homogenizer for approximately 5 min and flushed with centrifugation at 3500 × g for approximately 45 min to remove large debris. The supernatant was used for TAS and TOS analysis.

**Total antioxidant status (TAS)**

TAS was measured via the automated colorimetric measurement method Biotek Synergy HT plate reader immunostimulant gene 5 software and a Rel Assay (Rel Assay Diagnostics Kit, Mega Tip, Gaziantep, Türkiye) developed by Erel (Erel, 2004b). The hydroxyl radical is produced by the Fenton reaction and reacts with the colorless substrate, O-dianisidine, to produce the dianisyl radical, which is bright yellowish brown. Upon the addition of tissue samples, the oxidative reactions initiated by the hydroxyl radicals in the reaction mixture are suppressed by the antioxidant components of the sample, which prevents the color change from providing a measure of the total antioxidant capacity of the sample. The antioxidants in the sample increase the color in proportion to their concentration. The reaction can be monitored spectrophotometrically, and the opening ratio in color is proportional to TAS for the sample. The assay is calibrated with a stable antioxidant standard solution, which is traditionally referred to as Trolox equivalent, which is a vitamin E analog. The results are expressed as mmol Trolox equiv/L.

**Total oxidant status (TOS)**

TOS measurements were performed via a TOS Biotek Synergy HT plate reader with immunostimulant gene 5 software and a Rel Assay (Rel Assay Diagnostics Kit, Mega Tip, Gaziantep, Türkiye) developed by Erel. Oxidants in the tissue samples oxidize the ferrous ion–O-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules in the reaction medium. Ferric iron produces a colored compound with a chromogen in an acidic medium. This chromogen is orange and a reagent. The assay kit is calibrated in a similar manner. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated using $H_2O_2$, and the results are expressed as µmol $H_2O_2$ equiv/l.

**Oxidative stress index (OSI)**

The percent ratio of TOS to TAS is the oxidative stress index (OSI), an indicator of the degree of oxidative stress (Erel, 2004a, 2005). To perform the calculation, the resulting unit of TAS, mmol Trolox equivalent/L, was converted to µmol equivalent/L, and the OSI value was calculated via the following formula:

OSI (arbitrary unit) = [(TOS, µmol/l)/(TAS, mmol Trolox equivalent/l) × 100].

**Histopathology**

The tissue samples were fixed with 10% formaldehyde. After the tissue was subjected to follow-up procedures, the samples were dehydrated through 80%, 95% and absolute ethyl alcohol, cleared in xylene, and embedded in paraffin, and the tissue sections were cut into 4–5 µm thick paraffin

**Table 3.** Histopathological score findings

| Groups (n= 8) | İnfiltration | Epitelial damage |
|---|---|---|
| **Sham** | 0.0 (0.0-1.0) | 0.0 (0.0-2.0) |
| **PcE** | 0.0 (0.0-1.0) | 0.0 (0.0-2.0) |
| **CLP** | 0.0 (0.0-3.0)[a] | 0.0 (0.0-3.0)[a] |
| **PcE+CLP** | 0.0 (0.0-2.0)[b] | 0.0 (0.0-3.0) |

The results are expressed as the median (min–max). n= 8.
[a]*p*<.05 versus the Sham group. [b]*p*<.05 versus the CLP group.

**Table 4.** Average crypt debth, villus length (AM ± SD), severity of caspase-3 immunoreactivity and Paneth cell degranulation severity in each group.

| Groups (n= 8) | Crypt debth (µm) | Villi length (µm) | Caspase-3 immunoreactivity | Paneth cell degranulation severity |
|---|---|---|---|---|
| **Sham** | 166.25±36.02 | 260.52±50.05 | 6.0 (2.0-12.0) | 1.0 (0.0-3.0) |
| **PcE** | 165.90±37.49 | 281.55±42.46 | 6.0 (2.0-12.0) | 0.0 (0.0-3.0) |
| **CLP** | 147.05±36.41[a] | 237.13±49.70[a] | 3.0 (1.0-12.0)[a] | 2.0 (0.0-3.0)[a] |
| **PcE+CLP** | 175.02±34.33[b] | 264.61±49.84[b] | 6.0 (2.0-12.0)[b] | 1.0 (0.0-3.0)[b] |

AM ± SD: Arithmetic mean ± Standard deviation

The results are expressed as the median (min–max). n= 8.

[a]$p$<.05 versus the Sham group. [b]$p$<.05 versus the CLP group.

blocks. The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) (Bancroft & Gamble, 2008) for examination of morphology. Sections of the intestinal mucosa were examined for epithelial spillage and mononuclear cell infiltration. Alterations in structure were evaluated via a semiquantitative method according to the following scale: 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) (Taslidere et al., 2018). Twenty villus and crypt lengths were measured in each section. Additionally, 100 Paneth cells/section were evaluated for degree of degranulation (Adolph et al., 2013). Analyses were performed via the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK) with a Leica DFC-280 research microscope.

**Immunohistochemistry**

After deparaffinization and rehydration, the sections were placed in citrate buffer (pH 6.0) and antigen retrieval solution and boiled in a pressure cooker for 20 min. Then, the sections were washed with phosphate-buffered saline (PBS). After washing, 3% hydrogen peroxide solution was applied to block endogenous peroxide for 12 min at room temperature, after which the samples were washed with PBS. A protein blocker was applied to the sections to prevent nonspecific background staining. The sections were incubated with a caspase-3 primary antibody (rabbit polyclonal; Thermo Fisher Scientific, Anatomical Pathology, Fremont, USA) for 60 min, rinsed with PBS, and then incubated with biotinylated goat anti-polyvalent for 10 min and streptavidin-peroxidase for 10 min at room temperature. Staining was completed with chromogen (AEC substrate system; AEC chromogen and AEC substrate; 20 µl of AEC chromogen was added to 1 ml of AEC substrate) (Thermo Fisher Scientific, Anatomical Pathology, Fremont, USA) + substrate for 10 min, followed by counterstaining with Mayer's hematoxylin for 1 min, rinsing in tap water and dehydration. Caspase-3-positive cells were stained brown.

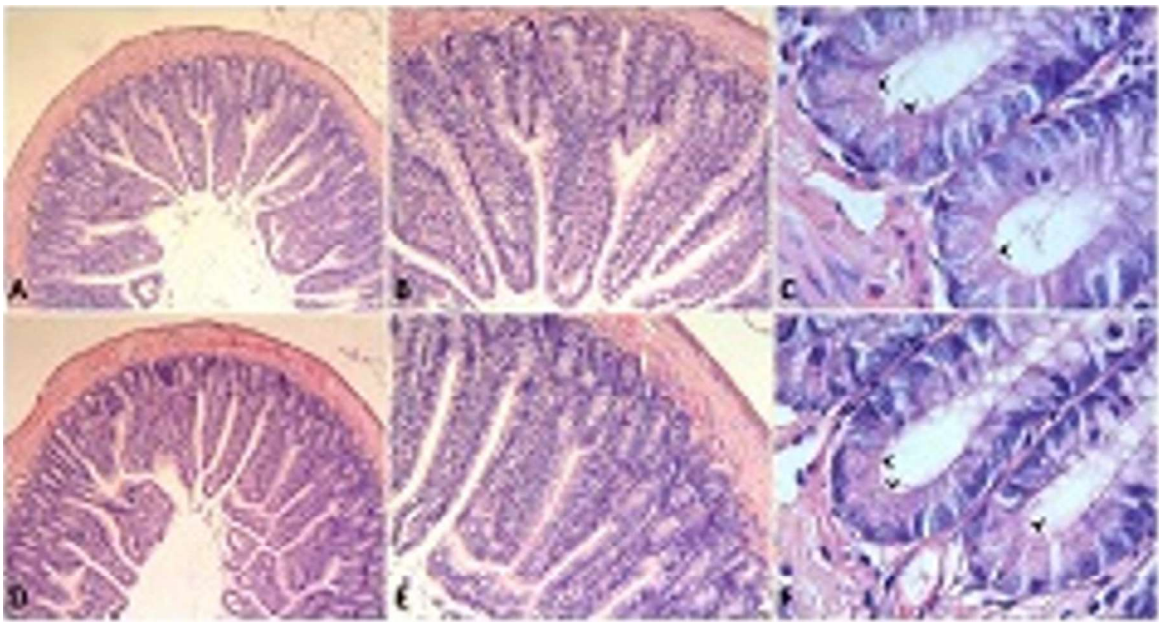

**Figure 1.** Paneth cells were characterized by eosinophilic granules located at the base of the intestinal tissue and crypts with a normal histological appearance in the Sham (A; 10×, B; 20×, C; 100×) and PcE (D; 10×, E; 20×, F; 100×) groups. Arrowheads indicate Paneth cells.

The sections were graded according to the degree of staining as follows: 1 = 0–25% staining; 2 = 26–50% staining; 3 = staining 51–75%; and 4 = staining 76–100%. The sections were graded according to their staining intensity as follows: 0 = no staining; 1 = weak but detectable staining; 2 = distinct; and 3 = intense staining. The total staining score was obtained as (prevalence)X(intensity) (Parlakpinar et al., 2019). All the sections were evaluated via a Leica DFC280 light microscope and a Leica Q Win image analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

**Statistical analyses.**

IBM SPSS statistics version 17.0 for Windows was used for the statistical analyses. Normally distributed data were analyzed by ANOVA (Tamhane). For nonnormally distributed data, comparisons between groups were performed with the Kruskal–Wallis H test. After the Kruskal–Wallis H test, multiple comparisons were made via the Conover test. The results are expressed as the median (minimum–maximum) and mean ± standard deviation. Values for $p \leq .05$ were considered statistically significant.

## Results

**Biochemistry**

Significant increases in the Hb, lymphocyte, neutrophil, BUN, Crea, ALT, AST and LDH levels were detected between the CLP, PcE+CLP and sham groups ($p<.05$), but no significant differences were detected between the CLP and PcE+CLP groups (Table 1).

Compared with those in the sham group, a significant increase in only the OSI was detected in the TOS, TAS and OSI in the CLP group ($p<.05$), but no significant difference in any parameter was observed between the CLP and PcE+CLP groups (Table 2).

**Histopathology**

The Sham and PcE groups presented a normal histological appearance of the intestinal mucosa, except for slight epithelial spillage and infiltration, which were observed in a few sections (Figure 1A, B and D, E). The crypta length was 166.25 ± 36.02 µm in the sham group and 165.90 ± 37.49 µm in the PcE group; the villus length was 260.52 ± 50.05 µm in the sham group and 281.55 ± 42.46 µm in the PcE group. Paneth cells were noted for the presence of densely packed eosinophilic granules in the Sham and PcE groups (Figure 1C, F).

The severity of epithelial spillage and infiltration was significantly greater in the CLP group than in the sham group ($p<.05$) (Figure 2A, B). In this group, the mean length of the crypta (147.05 ± 36.41 µm) and the length of the villus (237.13 ± 49.70 µm) were also significantly shorter

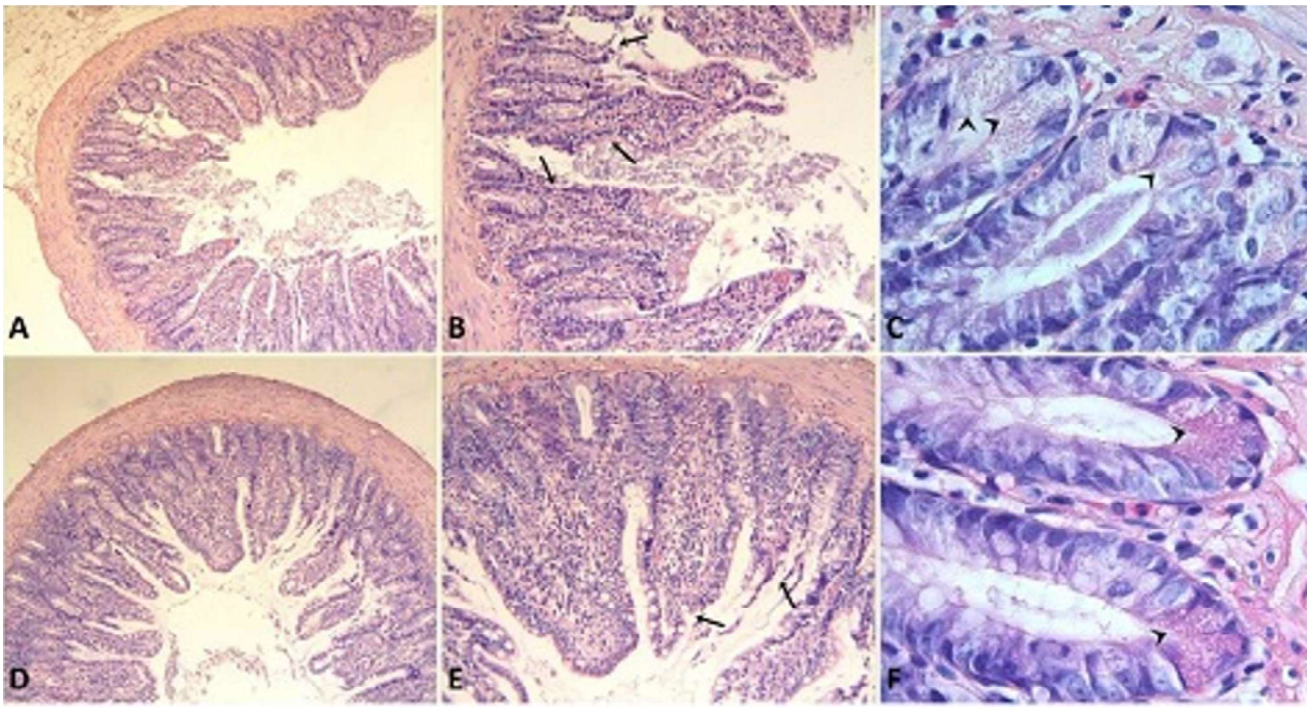


**Figure 2.** Appearance of villi, crypts and Paneth cells in the CLP (A; 10×, B; 20×, C; 100×) and PcE + CLP (D; 10×, E; 20×, F; 100×) groups. The arrows indicate epithelial damage, and the arrowheads indicate Paneth cells.

than those in the Sham group ($p<.05$). Notably, in the CLP group, Paneth cells had a hypertrophic appearance, and the granule content in their cytoplasm was significantly lower than that in the Sham group ($p<.05$) (Figure 2C, Table 4).

The severity of infiltration was significantly lower in the PcE+ CLP group than in the CLP group ($p<.05$); however, epithelial spillage in the PcE+ CLP group was similar to that in the CLP group (Figure 2D, E). In this group, the length of the crypta (175.02 ± 34.33 µm) and the length of the villus (264.61 ± 49.84 µm) were significantly greater than those in the CLP group ($p<.05$). The severity of Paneth cell degranulation was significantly lower in the PcE+CLP group than in the CLP group, and the appearance of Paneth cells was similar to that in the control group ($p<.05$) (Figure 2F, Table 4).

**Immunohistochemistry**

Caspase-3 immunoreactivity was observed in surface and gland epithelial cells. The intensity of caspase-3 immunoreactivity was similar in the sham and PcE groups (Figure 3A, B). In contrast, we detected decreased caspase-3 immunoreactivity in the CLP group compared with the sham group (P = 0.001) (Figure 3C). The intensity of caspase-3 immunoreactivity in epithelial cells was significantly greater in the PcE+CLP group than in the CLP group (P = 0.001) (Figure 3D). Caspase 3 expression levels are summarized in Table 4.

## Discussion

Sepsis is a complex, destructive condition accompanied by high mortality and limited treatment options. Despite improved surgical techniques and intensive care treatments, sepsis-related mortality rates remain high and continue to be a significant cause of hospital death (Iwashyna et al., 2012; Jones et al., 2008; Rodríguez et al., 2011). For these reasons, new and effective treatment methods for sepsis are highly important.

Multiple organ failure is the usual cause of death from sepsis. Lysosomal enzymes and free oxygen radicals, especially those released by neutrophils, participate in the pathogenesis of organ failure (Reinhart et al., 2005). Various antioxidants have been used to prevent the accumulation of free oxygen radicals, which contribute to mortality due to sepsis.

*İn vitro* and *in vivo*, extracts of Petroselinum crispum have been reported to possess antioxidant properties (Popović et al., 2007; Zhang et al., 2006); however, we have found no reports of its protective effects against sepsis. We

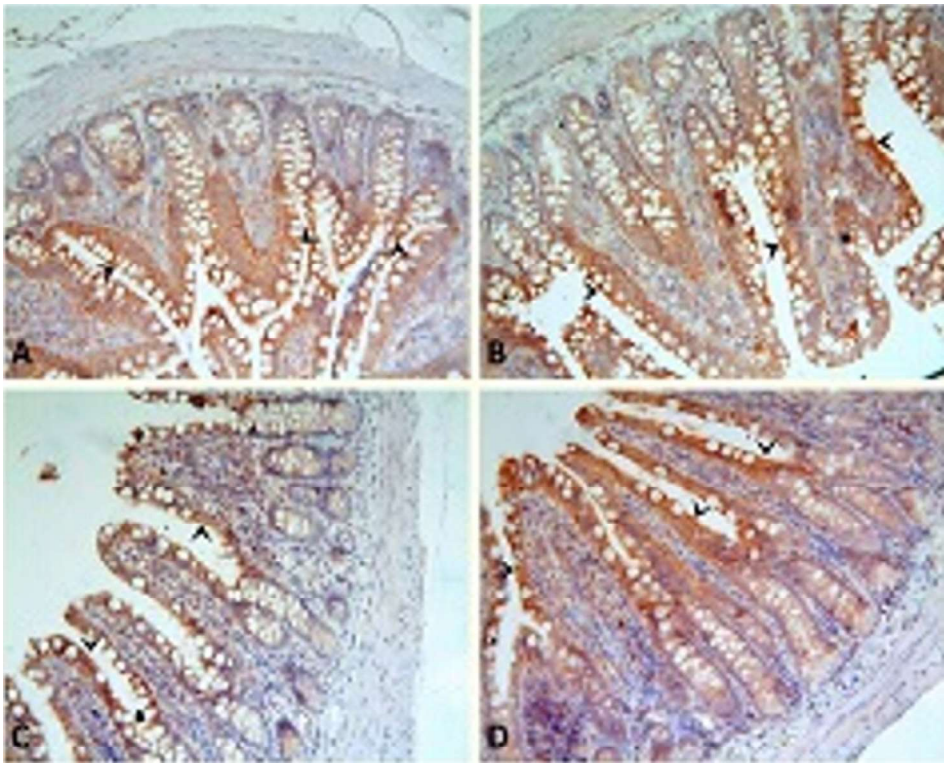


**Figure 3.** Similar caspase-3 immunoreactivity was observed in the sham (A) and PcE (B) groups. In the CLP group (C), a decrease in caspase-3 immunoreactivity was observed. On the other hand, a significant increase in caspase-3 immunoreactivity was noted with PcE treatment (D). Caspase-3 immunostaining; magnification ×20.

used CLP-induced sepsis as our model. Sepsis was verified by hemogram (neutrophils, lymphocytes), biochemical (AST, ALT, LDH, BUN, TAS, TOS and OSI levels) and histopathological changes, which are used to confirm sepsis. In the comparison between the sham and CLP groups, an increase in the OSI level indicates that CLP increases oxidative stress, whereas a decrease in the OSI between the CLP and PcE+CLP groups indicates that PcE reduces sepsis-induced oxidative stress.

There was no significant decrease in TAS in intestinal tissue extracts in the CLP group compared to the Sham group. The increase in the PcE+CLP group compared to the CLP group was not statistically significant. CT was significant in that it showed that CLP triggered sepsis and PcE reduced it., the increase in the TAS in the PcE+CLP group compared with that in the CLP group was not statistically significant, indicating that CLP induced sepsis, whereas PcE reduced it. Previous studies have shown that the levels of antioxidant markers are decreased in sepsis via CLP. In a study by Ritter et al., the activity of antioxidant enzymes such as catalase and superoxide dismutase was reduced in essential organs involved in the septic response (Ritter et al., 2004). reported an increase in the glutathione level in parsley extract. These findings suggest that parsley extract has a protective effect due to its antioxidant properties. These previous studies revealed that sepsis increases oxidative stress and that the activity of the antioxidant system decreases. In the present study, this situation was shown by the changes in TOS, OSI and TAS levels. We did not identify these genes individually but rather identified them as oxidative stress markers (TOS, OSI) and antioxidant markers (TAS).

Oxygen-free radicals, including malondialdehyde (MDA), hydrogen peroxide and hydroxyl radicals, cause peroxidative stress. Reduced antioxidative defenses, such as superoxide dismutase, catalase, and glutathione (GSH), also contribute to oxidative stress. Then, organ failure occurs (Koksal et al., 2004; Ozsoy-Sacan et al., 2006). In this study, parsley extract had a protective effect against CLP, with biochemical and histopathological analyses showing that it reduces multiorgan damage. This protective effect is primarily due to the inhibition of oxidative stress, which is one of the most important mechanisms of organ injury in sepsis. In our current study, the administration of parsley extract before CLP-induced sepsis increased neutrophil counts, PLTs and TASs, which decrease with sepsis, and decreased biochemical changes (BUN, AST, ALT, LDH, TOS, and OSI), which increase with sepsis, to protect against sepsis.

Histopathological changes revealed that epithelial damage and infiltration severity increased, whereas crypt–villus length, Paneth cell hypertrophy, and cytoplasmic granules decreased in the CLP group, suggesting sepsis syndrome. These changes have been confirmed by previous studies (Leng et al., 2014). The intestinal histological changes, including significantly reduced infiltration severity, were reversed by the use of PcE. This finding revealed the protective effect of PcE on the intestinal mucosa in sepsis. In the present study, we evaluated apoptosis via caspase-3 activity. Compared with that in the sham group, caspase-3 activity was significantly lower in the CLP group and significantly greater in the PcE + CLP group than in the CLP group, possibly because parsley extract has apoptosis-inducing activities and protective effects. The histopathological and biochemical results obtained in this study were similar to those of previous studies (Sęczyk et al., 2016; Shukla & Gupta, 2010).

As shown in previous studies, in this study, the decrease in the TOS and OSI levels, as well as their histopathological changes, especially a reduction in intestinal infiltration, demonstrated the antioxidant properties of PcE (Al-Juhaimi & Ghafoor, 2011; Fejes et al., 2000). In further research, we believe that the content of parsley extract will be elucidated in more detail via chromatographic methods and that the active substances or plant-prepared bioactive fractions isolated from the plant will offer alternative treatment options.

As a result, especially in hospitalized patients or other patients at risk for sepsis, we believe that our research may constitute the first step for the development of drugs that can reduce the risk of sepsis and facilitate treatment.

**Ethics Committee Approval:** The Ethics Committee for Experimental Animal Research approved our study (Reference no. 2015/A-34).
**Peer-review**: Externally peer-reviewed.
**Author Contributions:** Concept - H.P., Y.A.; Design- Y.F.C, A.P., H.P.; Supervision- H.P., Y.A.; Resources- Y.A., H.P.; Data Collection and/or Processing- O.O., I.K., N.V., K.T., A.K.A.; Analysis and/or Interpretation- O.O., I.K., N.V., K.T., A.K.A.; Literature Search- Y.A.; Writing Manuscript- Y.A.; Critical Review- H.P., Y.A.
**Conflict of Interest:** The authors have no conflicts of interest to declare.
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# Effect of Long-Term Use of Antithrombotics and Statins on COVID-19 Mortality and Clinical Severity

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**ABSTRACT**

**Objective:** Coronavirus Disease-2019 (COVID-19), has affected the whole world and is still an important disease with its mutations. In our study, we aimed to evaluate the effects of antithrombotic agents [acetylsalicylic acid (ASA), P2Y12 inhibitors, oral anticoagulants (OACs)] and statin treatments used before hospitalization on COVID-19 mortality and clinical severity.

**Methods:** A retrospective study was conducted on 5577 patients hospitalized with positive swab tests or findings consistent with COVID-19 on computed tomography. The 6-month mortality, in-hospital mortality, need for intensive care and intubation, and recurrent hospitalization outcomes of patients receiving chronic ASA (n=1210), P2Y12 inhibitors (n=357), OACs (n=1192), and statin (n=607) treatment were evaluated.

**Results:** The 6-month mortality rate was 13.5% (n=754), in-hospital mortality rate was 11.2% (n=627), the rate of admission to the intensive care unit was 16.1% (n=897), the need for intubation was 8.8% (n=493), and the rate of recurrent hospitalization was 10.4% (n=579). ASA and OACs reduced all outcomes. P2Y12 inhibitors provided benefit in other endpoints except intubation. Statins used before hospitalization did not provide a statistically significant decrease in 6-month mortality (p: 0.06), but were associated with a decrease in the rates of in-hospital mortality, need for intensive care, recurrent hospitalization, and intubation.

**Conclusion:** We found that long-term ASA, P2Y12 inhibitors, OACs and statin treatments used before hospitalization in patients hospitalized with COVID-19, reduced COVID-19 mortality and clinical severity. We think that these treatments may be beneficial in selected patient groups where post-COVID effects are observed.

**Keywords:** COVID-19, Antithrombotic, Statin, mortality

## Introduction

Coronavirus Disease-2019 (COVID-19) is a viral disease caused by Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2). It was considered a pandemic between 2020 and 2022 and can still cause clinically significant diseases with mutations (Cucinotta & Vanelli, 2020). COVID-19 has affected more than 775 million people worldwide and caused more than 7 million deaths (World Health Organization. Number of COVID-19 cases reported to WHO, 2024). Its effects have been observed in many systems, mainly the pulmonary system, as well as the immunological, gastrointestinal, cardiac and neurological systems (Zaim et al., 2020).

An increased risk of cardiovascular (CV) complications such as myocarditis, cardiac arrhythmias, and arterial and venous thrombosis has been reported in COVID-19 patients (Madjid et al., 2020). Additionally, underlying cardiovascular disease (CVD) and/or CV risk factors such as dyslipidemia, smoking, obesity; increase the risk of serious clinical complications and death in COVID-19 patients (Task Force for the management of, 2022).

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It has been shown that fatal complications of SARS-CoV-2 infection are due to an overactive inflammatory response resulting in cytokine storms (Hojyo et al., 2020). Although pneumonia and acute respiratory distress syndrome (ARDS) are the main complications of COVID-19, serious thrombotic complications have been reported in patients and are associated with increased mortality (Malas et al., 2020). COVID-19 is a prothrombotic state characterized by inflammation and elevations of D-dimer, fibrin/fibrinogen degradation products, and these laboratory parameters have been suggested as markers of severe disease and worse prognosis (Tang et al., 2020).

SARS-CoV-2 causes typical lymphocytic endotheliitis leading to widespread endothelial inflammation and dysfunction (Varga et al., 2020). Endothelial dysfunction causes a shift of the homeostatic balance toward a procoagulant state, leading to platelet adhesion and aggregation, thus initiating a thromboinflammatory process (Yau et al., 2015). Subsequently, ARDS and hypercoagulation occur with the release of inflammatory cytokines, thrombin production and fibrin clot deposition (Abou-Ismail et al., 2020). Microvascular and macrovascular thrombosis have been observed in both venous and arterial systems with COVID-19 and are the cause of poor prognosis. In addition, high thromboembolic events have been reported despite the use of prophylactic anticoagulation in COVID-19 patients with comorbid diseases (Nopp et al., 2020).

Currently, there is no specific pharmacological recommendation for the treatment of COVID-19, but antiviral, immunomodulatory, anti-inflammatory and antithrombotic agents are used. In our study, we aimed to evaluate the effect of long-term use of antiplatelet agents [acetylsalicylic acid (ASA), P2Y12 inhibitors] oral anticoagulants (OACs) (warfarin, dabigatran, apixaban, edoxaban, rivaroxaban) and statins on COVID-19 mortality and clinical severity.

## Methods

A total of 5577 patients who were admitted to the emergency department of Erzurum City Hospital between March and November 2020 and were diagnosed with COVID-19 by polymerase chain reaction (PCR) and/or computed tomography (CT), hospitalized, and started on treatment were included in our study. This study was conducted in accordance with the Declaration of Helsinki and with the approval of the local ethics committee. Ethics committee approval was received for this study from the ethics committee of Erzurum BEAH KAEK (Date: January 18, 2021, Number: 2021/02-33). Patient complaints, previous medical history, drug use history, clinical and demographic characteristics, hematological and biochemical parameters were identified from electronic medical records. Antiplatelet, anticoagulant, and statin treatments used by the patients in the last 1 year were recorded by identifying them in the National Medical Record System using the Social Security Institution website.

In accordance with the guidelines of the Ministry of Health of the Republic of Türkiye, COVID-19 diagnosis was made based on the patients' current complaints, contact history, physical examination findings, blood parameters, imaging findings, and PCR test results ("COVID-19 (SARS-CoV-2 ENFEKSİYONU) GENEL BİLGİLER, EPİDEMİYOLOJİ VE TANI ", 2020). Patients with a negative PCR test and no COVID-compatible findings on CT were not included in the study.

### COVID-19 Definition

Possible cases admitted to Erzurum City Hospital between March 2020 and November 2022 were evaluated according to the guidelines of the Ministry of Health of the Republic of Türkiye. Oral and nasal swab samples were taken from patients with suspected COVID-19, and polymerase chain reaction (PCR) was used for molecular analysis. Pulmonary computerized tomography (CT) was applied to selected patients deemed appropriate by the examining clinician ("COVID-19 (SARS-CoV-2 ENFEKSİYONU) GENEL BİLGİLER, EPİDEMİYOLOJİ VE TANI ", 2020). Complete blood count (CBC), inflammation parameters (C-reactive protein, ferritin) and biochemistry tests are routinely performed on patients all suspected patients with COVID-19. A second swab sample was taken from hospitalized patients when the first sample was negative. When one of the two samples taken was positive, the patient was diagnosed with COVID-19, and if both were negative, and the CT was negative, COVID-19 was excluded.

### ICU Admission Criteria

In the cases specified in the guideline, the patients were evaluated by the relevant clinician, and the need for intensive care was decided ("COVID-19 (SARS-CoV-2 ENFEKSİYONU) ERİŞKİN HASTA TEDAVİSİ," 2020).

- Patient with dyspnea and respiratory distress despite of oxygen therapy and in the follow-up, the oxygen requirement increased.
- Respiration rate ≥ 30/min
- $PaO_2/FiO_2 < 300$

**Table 1:** Baseline characteristics of patients

| Characteristics | All Patients (n= 5577) | Survivors (n=4823) | Nonsurvivors (n=754) | P Value |
|---|---|---|---|---|
| Age (year) | 61.4±16.4 | 59.5±16.2 | 74.1±11.3 | **<.001** |
| Gender (Male, %) | 2777 (49.8) | 2317 (48) | 460 (61) | **<.001** |
| HT (number, %) | 2760 (49.5) | 2274 (47.1) | 486 (64.5) | **<.001** |
| DM (number, %) | 1505 (27) | 1282 (26.6) | 223 (29.6) | .086 |
| CAD (number, %) | 1188 (21.3) | 963 (20) | 225 (29.8) | **<.001** |
| HF (number, %) | 294 (5.3) | 211 (4.4) | 83 (11) | **<.001** |
| COPD (number, %) | 765 (13.7) | 585 (12.1) | 180 (23.9) | **<.001** |
| CVD (number, %) | 125 (2.2) | 100 (2.1) | 25 (3.3) | **.032** |
| AF (number, %) | 368 (6.6) | 247 (5.2) | 121 (16.1) | **<.001** |
| HL (number, %) | 686 (12.3) | 599 (12.4) | 87 (11.5) | .501 |
| CRF (number, %) | 146 (2.6) | 104 (2.2) | 42 (5.6) | **<.001** |
| Asthma (number, %) | 228 (4.1) | 207 (4.3) | 21 (2.8) | .052 |
| **Medications** | | | | |
| ASA (number, %) | 1210 (21.7) | 1013 (21) | 197 (26.1) | **.001** |
| P2Y12 inhibitors (number, % | 357 (6.4) | 295 (6.1) | 62 (8.2) | **.028** |
| Anticoagulants (number, %) | 1192 (21.4) | 1092 (22.6) | 100 (13.3) | **<.001** |
| Statins (number, %) | 607 (10.9) | 510 (10.6) | 97 (12.9) | .060 |
| ACEI / ARB (number, %) | 1908 (34.2) | 1601 (33.2) | 307 (40.7) | **<.001** |
| BB (number, %) | 1159 (20.8) | 972 (20.2) | 187 (24.8) | **.003** |
| CCB (number, %) | 972 (17.4) | 792 (16.4) | 180 (23.9) | **<.001** |
| Diuretics (number, %) | 1682 (30.2) | 1399 (29) | 283 (37.5) | **<.001** |
| **Laboratory** | | | | |
| Hb (g/dL) | 13.3 (12.1-14.4) | 13.4 (12.3-14.4) | 12.3 (10.4-14.3) | **<.001** |
| Htc (%) | 41.1 (37.8-44.2) | 41.2 (38.2-44.1) | 39 (33.3-44.9) | **<.001** |
| Wbc ($10^3$/µL) | 7.21 (5.53-9.55) | 6.93 (5.36-8.93) | 10.45 (7.61-13.87) | **<.001** |
| Neutrophil count ($10^3$/µL) | 5.18 (3.58-7.49) | 4.82 (3.43-6.82) | 8.95 (6.13-11.89) | **<.001** |
| Lymphocyte count ($10^3$/µL) | 1.23 (0.86-1.68) | 1.30 (0.95-1.73) | 0.71 (0.49-1.03) | **<.001** |
| Platelet count ($10^3$/µL) | 231.7 (184-289.2) | 238 (190.8-295) | 190.6 (144.4-242.4) | **<.001** |
| AST (U/L) | 33 (24.5-47.5) | 31.3 (23.7-43.5) | 53.4 (34.8-101) | **<.001** |
| ALT (U/L) | 31.3 (21-49.5) | 31 (21-47.5) | 35.3 (21.8-70.7) | **<.001** |
| Ferritin, (ng/mL) | 257.4 (111.4-547.7) | 229.1 (98.1-467.1) | 595.9 (280.5-1119.5) | **<.001** |
| LDH (U/L) | 297.6 (236-384) | 283.1 (229.5-353.5) | 482 (368.4-625.8) | **<.001** |
| CRP (mg/L) | 36.8 (12.8-75.7) | 30.8 (10.3-62.5) | 95.6 (53.1-145.9) | **<.001** |
| D-dimer (µg/mL) | 384 (104-1318) | 302 (91-956) | 2540 (768.5-7500) | **<.001** |
| Procalcitonin (ng/mL) | 0.11 (0.02-0.57) | 0.08 (0.01-0.35) | 1.07 (0.335-3.32) | **<.001** |
| pH level | 7.40 (7.38-7.44) | 7.40 (7.37-7.43) | 7.44 (7.40-7.45) | **.046** |
| Lactic acid (mmole/L) | 2.1 (1.6-2.7) | 2 (1.6-2.6) | 2.75 (2.1-3.6) | **<.001** |
| Troponin I (ng/mL) | 0.011 (0.003-0.1) | 0.008 (0.002-0.033) | 0.265 (0.057-1.375) | **<.001** |
| NTproBNP (pg/mL) | 444 (83.8-4916.5) | 159 (46.2-702.5) | 7074 (1704-20911.7) | **<.001** |
| Creatinine (mg/dL) | 0.88 (0.73-1.12) | 0.85 (0.72-1.06) | 1.28 (0.94-1.95) | **<.001** |
| Na (mmol/L) | 137 (134.6-139.1) | 136.7 (134.5-138.9) | 139.5 (135.8-143.9) | **<.001** |
| K (mmol/L) | 4.18 (3.89-4.49) | 4.15 (3.88-4.44) | 4.40 (4.03-4.85) | **<.001** |
| Glucose (mg/dL) | 131 (136-179.3) | 126.7 (104-172) | 162.2 (129.1-208) | **<.001** |
| Albumin (g/L) | 3.8 (3.4-4.1) | 3.8 (3.5-4.1) | 3.15 (2.86-3.52) | **<.001** |
| INR | 1.07 (1-1.18) | 1.07 (1-1.18) | 1.08 (1-1.20) | .153 |
| TG (mg/dL) | 128 (94.3-179) | 126.5 (93.3-126.8) | 138.3 (104.2-192.5) | **<.001** |
| HDL (mg/dL) | 33.5 (27.2-41.2) | 34.1 (28-41.7) | 28.6 (22.4-36.6) | **<.001** |
| LDL (mg/dL) | 86 (67-109) | 88 (69.4-110.8) | 72.5 (54.3-96.5) | **<.001** |
| COVID-19 PCR test (positive, %) | 3809 (68.3) | 3300 (68.4) | 509 (67.5) | .615 |
| Lung involvement in tomography (positive, %) | 4753 (85.2) | 4075 (84.5) | 678 (89.9) | **<.001** |

**Abbreviations:** HT: hypertension, DM: diabetes mellitus, CAD: coronary artery disease, HF: heart failure COPD: chronic obstructive pulmonary disease, CVD: cerebrovascular disease, AF: atrial fibrillation HL: hyperlipidemia, CRF: chonic renal failure, ASA: acetylsalicylic acid ACEI: angiotensin converting enzyme inhibitor, ARB: angiotensin reseptor blocker, BB: beta blocker, CCB: calcium channel blockers, Hb: haemoglobin, Htc: hematocrit, Wbc: white blood cell, AST: aspartate aminotransferase, ALT: alanine aminotrasferase, LDH: Lactate dehydrogenase, CRP: C-reactive protein, NTproBNP: N-terminal probrain natriuretic peptide, Na: sodium, K: potassium, INR: International Normalized Ratio, TG: triglycerides, HDL: high density lipoprotein, LDL: low density lipoprotein, PCR: polymerase chain reaction

- SpO2 < 90% or PaO2 < 70 mmHg despite 5 L/min oxygen therapy
- Hypotension (systolic blood pressure < 90 mmHg and more than 40 mmHg decrease from normal systolic blood pressure and mean arterial pressure < 65 mmHg, tachycardia > 100/min
- Patients with acute kidney injury, acute liver function tests, confusion, acute organ dysfunction such as acute bleeding diathesis and immunosuppression
- Troponin elevation and arrhythmia
- Lactate > 2 mmol
- Presence of skin disorders such as capillary return disorder and cutis marmaratus

The study endpoints were determined as 6-month mortality, in-hospital mortality, need for intensive care, need for intubation, and re-hospitalization, and the results were retrospectively obtained from electronic medical records.

**Statistical analysis**

Data were analyzed using the SPSS 23.0 version (IBM, Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation or median (interquartile range), and categorical variables were expressed as percentages. Whether continuous variables fit the normal distribution was determined using the "Homogeneity of Variance" test. Continuous variables were compared using "Student's t-test" and "Mann–Whitney U test" as appropriate. Categorical variables were compared using the "chi-square" test. Variables were considered statistically significant when the *p* value was <.05.

## Results

The mean age of 5577 patients included in our study was 61.4 ± 16.4 years and 49.8% were male. PCR test was positive in 3809 (68.3%) patients and COVID-19 findings were observed in computed tomography of 4753 (85.2%) patients. The most common comorbidity in patients was hypertension (n=2760 (49.5%). Mortality was observed in 754 patients; these patients were older (74.1 ± 11.3) and 61% were male. Among those with mortality, the most common comorbidity was hypertension with 64.5%. D-Dimer, troponin, crp, ferritin, NTproBNP were higher in patients with mortality. Baseline demographic data of the study population are shown in Table-1.

Before admission, 21.8% of the patients were using ASA, 24.4% were using OACs, 6.4% were using P2Y12 inhibitors and 1.9% were using statins (Figure-1). In addition, the rates of other medications used by the patients before admission are shown in Table-1.

When the study endpoints were examined, the 6-month mortality rate was 13.5%, the in-hospital mortality rate was 11.2%, the rate of patients need intensive care was 16.1%, the rate of patients intubated was 8.8%, and the rate of recurrent hospitalization was 10.4% (Table 2).

According to the study results, ASA and OACs used before hospitalization reduced the 6-month mortality, in-hospital mortality, need for intensive care, recurrent hospitalizations and need for intubation outcomes. P2Y12 inhibitors provided benefit in other endpoints except intubation. Statins used before hospitalization did not provide a significant reduction in 6-month mortality (p: 0.06) but were associated with a reduction in in-hospital mortality, need for intensive care, recurrent hospitalizations and intubation rates (Table 3).

**Table 2:** Course of the COVID-19 disease

| | **All Patients (n = 5577)** |
|---|---|
| **Total mortality (number, %)** | 754 (13.5) |
| **In-hospital mortality (number, %)** | 627 (11.2) |
| **Need for intensive care (number, %)** | 897 (16.1) |
| **Need for intubation (number, %)** | 493 (8.8) |
| **Recurrent hospitalization (number, %)** | 579 (10.4) |

## Discussion

**Acetylsalicylic acid (ASA)**

Acetylsalicylic acid (ASA) inhibits platelet functions by irreversibly inhibiting cyclooxygenase (COX) activity. Low doses show an antithrombotic effect by inhibiting the formation of thromboxane A2 by acetylation of COX-1, while higher doses block prostaglandin production by inhibiting COX-1 and COX-2, resulting in analgesic, anti-inflammatory and antipyretic effects (Pillinger et al., 1998). ASA also has antiviral and immunomodulatory effects. Studies have shown that ASA reduces the production of interleukin-6 (IL-6), C-reactive protein (CRP) and macrophage colony stimulating factor (Glatthaar-Saalmuller et al., 2017; Ikonomidis et al., 1999). Additionally, ASA has been associated with prevention of ARDS and protection from acute lung injury and reduced mortality (Boyle et al., 2015; Panka et al., 2017). Researchers have suggested ASA and other antiplatelet therapies as potential agents in COVID-19 due to the high burden of microvascular thrombosis (Bikdeli et al., 2020). Similarly, we think that the beneficial effects of aspirin in our study are due to its anti-inflammatory, analgesic,

antithrombotic, antipyretic, antiviral and immunomodulatory effects.

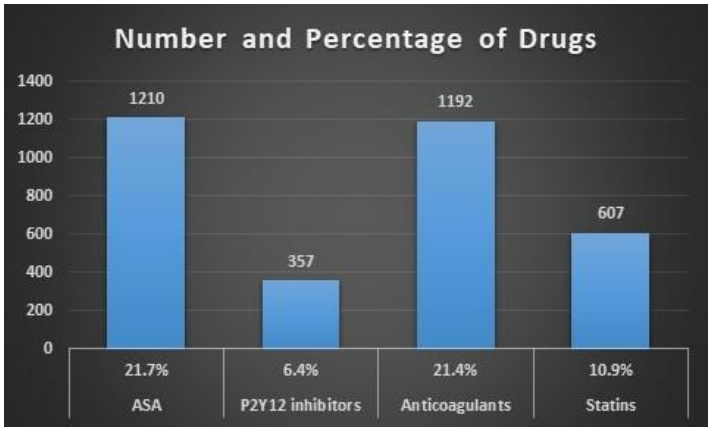


**Figure 1:** Drug use in COVID-19 patients

**P2Y12 Inhibitors**

Researchers have suggested antiplatelet treatments in COVID-19 patients due to the high burden of thrombosis (Bikdeli et al., 2020). Additionally, antiplatelet therapy has been shown to have beneficial effects on ARDS, acute lung injury, sepsis and mortality (Du et al., 2018; Wang et al., 2016). However, there are many unknowns regarding the use and benefits of P2Y12 inhibitors in COVID-19. It is not known which stage of the disease will respond best, nor is it known what agent and dose are most appropriate to provide maximum effect while reducing bleeding risks. In addition, thrombocytopenia is associated with an increased risk for worse clinical outcomes with COVID-19 (Lippi et al., 2020). It has been recommended that antiplatelet therapy be used with caution in the presence of clinical conditions such as concomitant coronary artery disease and acute coronary syndrome in COVID-19 patients (Bikdeli et al., 2020). Similarly, in our study, we observed less mortality and better clinical status in patients using P2Y12 inhibitors.

**Anticoagulants**

Thromboprophylaxis with anticoagulants, particularly low molecular weight heparin (LMWH), has been shown to be beneficial in reducing the risk of COVID-19 complications and mortality and is recommended for all hospitalized patients with COVID-19 ("COVID-19 (SARS-CoV-2 ENFEKSİYONU) ERİŞKİN HASTA TEDAVİSİ," 2020; Moores et al., 2020). The use of vitamin K antagonists and direct oral anticoagulants (DOACs) in the treatment of COVID-19 is controversial, and switching from oral anticoagulation to LMWH is recommended in patients hospitalized with COVID-19 infection (Moores et al., 2020; Testa et al., 2020). Various studies have been conducted on the effects of anticoagulant treatments used before hospitalization on COVID-19 outcomes, and conflicting results have been observed. While there are studies that show no difference in clinical outcomes between patients treated with VKAs and DOACs (Spiegelenberg et al., 2021), there are also studies that independently associate long-term oral anticoagulation with DOACs or VKAs with better outcomes (Frohlich et al., 2021). In our study, although it is not clearly known whether these drugs were continued during the hospitalization of patients using chronic OACs and it is thought that they were probably switched to LMWH, we observed that there was a relationship between mortality and the severity of COVID-19 in patients using chronic OACs.

**Statins**

Statins are lipid-lowering drugs and used to prevent CV events (Grundy et al., 2019). The effects of statins on COVID-19 are unclear. Some studies report no difference in mortality and severe infection outcomes, while others even show adverse outcomes in statin users compared with non-users (Butt et al., 2020; Hariyanto & Kurniawan, 2020, 2021). However, many studies have found that statin use is associated with a reduced risk of mortality, reduced risk of adverse outcomes, reduced disease severity, and reduced recovery time in COVID-19 patients (Cariou et al., 2021; Kow & Hasan, 2020; Pal et al., 2022). Overall, there were more arguments in favor of continuing use rather than interrupting statin therapy in patients with COVID-19. In our study, although the total mortality was low in number, no statistically significant difference was observed. This

**Table 3:** Effects of antithrombotic drugs and statins on the course of the disease

| | 6-months Mortality | In-hospital Mortality | Need for Intensive Care | Need for Intubation | Recurrent Hospitalization |
|---|---|---|---|---|---|
| **ASA** | **0.001** | **0.001** | **<0.001** | **0.021** | **<0.001** |
| **P2Y12 inhibitors** | **0.028** | **0.026** | **<0.001** | 0.152 | **<0.001** |
| **OACs** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** |
| **Statins** | 0.060 | **0.023** | **<0.001** | **0.013** | **<0.001** |

Abbreviations: ASA: acetylsalicylic acid, OACs: oral anticoagulants

situation can be explained by the relatively low number of statin use. Additionally, in our study, a decrease in in-hospital mortality and COVID-19 severity was observed in patients using statins.

### Post-COVID Syndrome

Post-COVID syndrome has been defined as signs and symptoms that develop during or after COVID-19 infection, are present for more than 12 weeks, and cannot be attributed to alternative diagnoses (Shah et al., 2021). The most common symptoms are fatigue and breathlessness. Symptoms may be singular, multiple, constant, transient, or fluctuating, and canchange in natüre over time. Potential mechanisms contributing to the pathophysiology of post-COVID may be driven by tissue damage caused by virus-specific changes or long-lasting inflammatory response, immune dysregulation, and autoimmune reactions. Currently, management options are limited because there is insufficient data of post-COVID. It must be a personalized approach involving monitoring ongoing symptoms and late complications, symptomatic treatment, palliative care, physical rehabilitation, mental health, and psycho-social support. In a personalized approach in post-COVID patients, the use of antithrombotics and statins can be considered, especially in cases of cardiac complications.

### Limitations

Our study has some limitations common to retrospective studies. Data on the duration and dosage of medications used before admission and whether they were continued during hospitalization are limited. We have limited data regarding the anticoagulation dose that patients receive while in the intensive care unit. We also did not evaluate the interaction of these drugs with other potential treatments for COVID-19, such as antiviral drugs, or the impact of in-hospital interventions that may affect outcomes. Our study evaluated hospitalized patients and cannot be generalized to asymptomatic or symptomatic non-hospitalized patients. Also, since it is a single center study, its generalization to the entire population is limited.

## Conclusions

We observed that long-term ASA, P2Y12 inhibitors, OACs and statin treatments used before hospitalization in patients hospitalized with COVID-19, reduced COVID-19 mortality and clinical severity. We think that these drugs can be given to selected individuals in patient groups defined as post-COVID and with ongoing active complaints.

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# The Effect of Amygdalin on Glioblastoma: Focus on Oxidant Capacity and Antioxidant Status

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**ABSTRACT**

**Objective:** Amygdalin (Vitamin B-17) is a type of vitamin, naturally found in many fruits and plants. The aim of the study was the evaluation of Amygdalin effect on the oxidant capacity and oxidant status of the T98G cancer cells. A T98G cell line was used in the study. Cell viability and oxidative stress evaluation were done.
**Methods:** Amygdalin was used at 1, 4, and 8 µg/mL doses. TAC and TOS values were measured.
**Results:** According to the result, amygdalin 8 µg/mL shows the highest anticancer effects. TAC level was 3.2 Trolox Equiv/L and TOS was 3.6 H2O2 Equiv/L.
**Conclusion:** Vit B17 can increase oxidative stress in T98G cells and decrease cell viabilit

**Keywords:** Amygdalin, MTT, TAC, TOS, T98G

## Introduction

Glioblastoma multiforme (GBM) is one of the aggressive types of brain cancer. It is also the most common malignant primary tumor of the brain and central nervous system (Altinoz et al., 2022; Yeni et al., 2023). It accounts for 14.5% of all central nervous system tumors and 48.6% of malignant central nervous system tumors. Cancer treatment is traditionally done with chemotherapy, surgery, or a combination of these. Despite medical advances today, treatment options and success rates are low. Therefore, it is essential to develop or search new substances for brain tumors (Kafagi et al., 2024; Loginova et al., 2024). Our study aims to evaluate the effect of Amygdalin on the glioblastoma line and oxidative stress parameters. Amygdalin or vitamin B17 is naturally found in many plants, fruits, and seeds.

Vit B17 has many effects. Some of them are; antitussive, antiasthmatic, fibrosis prevention, anticancer, anti-inflammatory, and anti-ulcer activities (El-Desouky et al., 2020; Tousson et al., 2020). In our study, TAC and TOS tests as well as viability rate will be evaluated to determine anticancer activity.

## Method

### Chemicals and reagents

All of the reagents were of analytical grade and used without further purification. Amygdalin (Vit B17, Gloden Pharm,Kyiv, Ukraine). Dulbecco-modified eagle medium (DMEM), fetal bovine serum (FBS), Antibiotic, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA).

### T98G cell culture

The T98G cell line was obtained from Bilecik University's Department of Medical Pharmacology. Cells were centrifuged at 1200 rpm for 5 minutes. They were suspended in fresh Dulbecco-modified eagle medium-F12 (DMEM), fetal bovine serum (FBS) 10%, and antibiotics 1% (penicillin, streptomycin, and amphotericin B). 48-well plates (5% CO2; 37 °C)

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were used for seeding.

**MTT assay**

Briefly, cells were resuspended in fresh DMEM medium, 10% FBS, and 1% antibiotic (penicillin, streptomycin, and amphotericin B). Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL were administered for 24 hours. The optical density of the solutions was read at 570 nm using a Multiskan™ GO microplate spectrophotometer (Thermo Fisher, Porto Salvo, Portugal).

**TAC (total antioxidant capacity) and TOS (total oxidant status)**

TAC level was measured by using the Rel Assay Total Antioxidant Capacity (Rel Assay Diagnostics, Gaziantep, Türkiye) commercial kit. Briefly, the supernatant was used and the reagent was added according to the manufacturing protocol. The color change was evaluated by measuring at a wavelength of 660 nm wavelength for TAC and 530 nm wavelength for TOS. TAC Results were expressed per µmol Trolox Equiv/L. TOS results are expressed per µmol H2O2 Equiv/mg protein.

**Statistical analysis**

The results were analyzed with SPSS 20.0 (IBM SPSS Corp., Armonk, NY, USA) Windows program and given as mean and standard error. Statistical comparison tests were performed between groups using the One Way Anova test, and data with a significance of $p<.05$ were considered statistically significant.

## Results

**MTT assay**

MTT results regarding cell viability are shown in Figure 1. In this study, the 24-hour exposure results of the groups Control, Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL were evaluated. The control group was rated as 100 and the viability rate of the other groups was proportioned and compared to the control group. Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL cell viability ratios were 89, 83, and 75 respectively. It was determined that Amygdalin 8 µg/mL was significantly different from the control at a rate of $p<.05$.

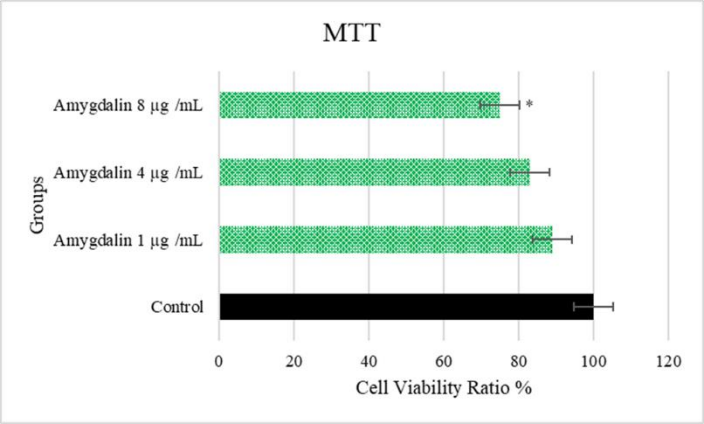


**Figure 1.** The cell viability ratio of T98G 24 hours after treatment. The experimental groups contain the Control group, Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL. Statistical significance: * $p<.05$ compared to the control group.

**TAC and TOS assay**

AK Evaluation results regarding TAC and TOS results are shown in Figure 2. TAC and TOS evaluations resulting from 24-hour exposure to Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL were determined.

TAC: It is observed that the use of Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL decreased TAC values. Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL show statistically differences of 3.5 and 3.2 respectively ($p<.001$).

TOS: Amyigdalin increased TOS value. At doses of Amygdalin 4 µg/mL $p<.05$, and Amygdalin 8 µg/mL $p<.001$ statistically significant differences were obtained.

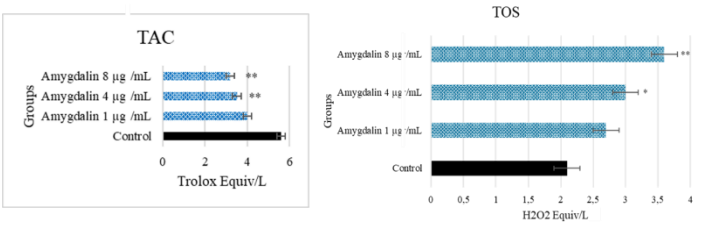


**Figure 2.** The Total Antioxidant Capacity and Total Oxidant Capacity of T98G cell lines 24 hours after treatment. The experimental groups contain the Control group, Amygdalin 1 µg/mL, Amygdalin 4 µg/mL, and Amygdalin 8 µg/mL. Statistical significance: * $p<.05$, ** $p<.001$ compared to the control group.

## Discussion

In recent years, scientific research has been conducted

to investigate the effectiveness and safety of Amygdalin (Vit B17) against many types of cancer. Although in vitro studies are widely conducted on prostate, digestive system, and breast cancer, glioblastoma studies are limited. Cell cycle disruption, apoptosis, and toxicity due to oxidative stress have been shown in some studies (Jaszczak-Wilke et al., 2021; Kolesarova et al., 2021). However, the study on the level of TAC and TOS levels on the T98G cell line is presented for the first time with this publication.

A study has shown that Amygdalin can induce apoptosis in the human promyelocytic leukemia (HL-60) cell line (Kwon HeeYoung et al., 2003). Another study with SNU-C4 cells has shown that cell cycle and proliferation are suppressed with amygdalin (Kwon HeeYoung et al., 2003; Lin et al., 2022). In the current study, it has been shown that the use of Amygdalin reduces the viability of cancer cells depending on the dose. In addition, the most effective dose was determined as 8 µg/mL. Although this effect is consistent with the literature, the point that should be noted is the amygdalin concentration. Although the effect starts with a dose of 4 µg/mL, significant change and death occur when this dose is doubled. When the total antioxidant and oxidant levels are examined in the continuation of the mechanism review, it is understood that the decrease in TOC value means that the protective mechanism of cancer is disabled and oxidants could have damaged mitochondrial organelles (Makarević et al., 2016). Although amygdalin is only related to the rhodenase enzyme, which causes death in cancer cells as a mechanism, studies (Systemic-Review 2015) have shown that amygdalin is beneficial in cancer patients (Milazzo et al., 2006; Song & Xu, 2014). In addition, studies conducted with HeLa, DU145, and LNCaP cells have shown that amygdalin is effective on Bax and Bcl-2 gene expressions, as well as an increase in cellular stress (Milazzo et al., 2006; Park et al., 2005; Seyhan et al., 2023). In vitro experiments have shown that Amygdalin induces apoptosis by increasing the expression of Bax protein and caspase-3 and decreasing the expression of the antiapoptotic BcL-2 protein (Fernald & Kurokawa, 2013; Carter et al., 1980; Lee & Moon, 2016). Amygdalin also reduces the expression of integrins. Accordingly, it reduces catenin levels and consequently inhibits the metastasis of cancer cells. In addition, studies have shown that it inhibits the adhesion of breast cancer cells, lung cancer cells, and bladder cancer cells by inhibiting the Akt-mTOR pathway. Amygdalin increased the expression of the p19 protein in kidney cancer cells. As a result, it led to the inhibition of cell transfer from the G1 phase to the S phase and thus inhibited cell proliferation (Gogolin et al., 2013; Makarević et al., 2014; Krebs, 1970). Amygdalin also inhibited the NF-kβ signaling pathway and showed anti-inflammatory activity by affecting the release of proinflammatory cytokines (Krebs, 1970**;** Bauernfeind et al., 2009). In our study, it was determined that oxidative stress increased in the cancer line after cancer cell exposure to amygdalin and, conversely, antioxidant capacity decreased.

## Conclusion

In conclusion, our study showed that Amygdalin has a cytotoxic effect on glioblastoma cancer cells by causing oxidative stress. Based on the information in the literature, this effect is likely to occur through an apoptotic mechanism. Our results suggest that Amygdalin can be considered as an adjuvant treatment in GBM cancer patients. However, further studies are needed to investigate the exact mechanism of the effect.

**Ethics Committee Approval:** Since it is a cell culture study, ethics committee approval is not required.
**Peer-review**: Externally peer-reviewed.
**Author Contributions:** Concept – S.G.; Design - S.G.; Supervision – S.G., A.T.; Resources – K.K.; Materials – E.N., S.G.; DA.T.a Collection and/or Processing – YEM, A.T.; Analysis and/or Interpretation – A.T., B.A.; Literature Search – S.G., A.E.A.; Writing Manuscript – K.K., S.G.; Critical Review – A.T., E.N.; Other – A.T..
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# Relationship Between Oxidative Stress and Cellular Adenosine Triphosphate Levels

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## ABSTRACT

Oxidative stress (OS) refers to the deterioration of the balance between oxidants and antioxidants in favor of oxidants, and this may lead to disruptions in redox signaling and control and/or damage at the molecular level. The presence of low levels of reactive oxygen species (ROS) plays a physiological role in intracellular signaling pathways. However, damage may occur in cells and tissues as a result of excessive increase in ROS production. Because ROS have the potential to damage almost all structures in the cell, including lipid, protein, deoxyribo nucleicacid (DNA). The main source of free radicals in the cell is mitochondria. ROS formation is a natural consequence of oxidative phosphorylation resulting in adenosine triphosphate (ATP) production in mitochondria. The attack of these radicals results in damage to the mitochondria, a decrease in the activity of oxidative phosphorylation enzymes and consequently a decrease in ATP synthesis. On the other hand, ATP is needed for antioxidant synthesis, which is necessary for cell defence against increasing ROS. Therefore, a decrease in ATP levels makes tissues vulnerable to OS. In this case, it is likely that tissues exposed to OS will also have problems in ATP production and the decrease in ATP synthesis will further increase oxidative damage.

**Keywords:** Reactive oxygen species, Adenosine triphosphate, Mitochondria, Antioxidant defense systems

## Introduction

Under physiological conditions, tissues and organs have a tightly regulated and highly dynamic redox balance to maintain the balance between oxidants and antioxidants. However, the capacity of the endogenous antioxidant system is exceeded when the formation of reactive oxygen species (ROS) increases for a variety of reasons (Sthijns et al., 2018). This is known as oxidative stress (OS), which leads to impaired redox signalling and control and/or molecular damage (Sthijns et al., 2018). The concept of OS was first officially articulated by Sies in 1985. It continues to be the centre of attention since that day (Sies, 2018).

The presence of low levels of ROS plays a physiological role in intracellular signaling pathways (Kowalczyk et al., 2021). However, in the presence of OS, ROS targets almost all structures in the cell including lipids, proteins, and deoxyribo nucleicacid (DNA). Lipids are the most sensitive structures against ROS oxidation. The reaction of polyunsaturated fatty acids, especially arachidonic acid and docosahexaenoic acids with ROS causes oxidative degradation (Pisoschi and Pop, 2015). As a result of this degradation, by-products such as malondialdehyde (MDA), which is often used to determine the presence of OS as a lipid peroxidation by-product covalently bound to cellular proteins are produced (De Cristóbal et al., 2002; Schütt et al., 2012). ROS, which also targets proteins, causes oxidation of both the backbone and side chains of proteins. Nucleic acids are also targeted by ROS attacks, which can result in DNA-protein cross-linking, strand breaks, and DNA mutations (Pisoschi and Pop, 2015). These attacks result in the formation of modified bases such as 8-hydroxydeoxyguanosine (8OHdG), which is also used as an OS parameter (Schütt et al., 2012).

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The oxidative attack of ROS has been the main subject of numerous studies from the past to the present, and as a result, OS has been observed to play a role in the pathogenesis of many diseases (Ghezzi et al., 2017). For example, oxidative DNA damage has been shown to lead to oncogene activation and thus to tumour formation and/or carcinogenesis (Pramanya & Alı, 2019). In the literature, OS has also been implicated in the etiology of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Gandhi and Abhramov reported an increase in lipid peroxidation products, protein carbonylation, and hydroxylated guanine in both diseases and drew attention to the role of mitochondrial dysfunction in the development of neurodegenerative diseases (Gandhi and Abramov, 2012). It has been suggested that ROS leads to atherosclerotic lesions and lipid accumulation through oxidative changes in low-density lipoproteins (Pramanya & Alı, 2019). It has also been reported that inflammation is triggered by ROS-induced nuclear factor-kappa B activation and is responsible for developing inflammatory diseases such as rheumatoid arthritis (Pramanya & Alı, 2019).

ROS, which are involved in both physiological and pathological processes, are produced as by-products of aerobic respiration and various catabolic and anabolic processes (Liang et al., 2007; Pisoschi and Pop, 2015; Kowalczyk et al., 2021).. The main source of free radicals in the cell is mitochondria. The formation of ROS is mainly a natural consequence of oxidative phosphorylation resulting in the production of adenosine triphosphate (ATP). In this process, approximately 1-2% of the molecular oxygen normally used by cells is converted into ROS. Mitochondria, the source of both ATP synthesis and ROS production, have a double membrane, outer (separating them from the cytosol) and inner. Oxidative phosphorylation occurs in the inner mitochondrial membrane, which contains four large enzyme complexes. The energy released in the reactions in the electron transport chain in this membrane is used for ATP synthesis (Kowalczyk et al., 2021). ATP is a nucleoside triphosphate composed of a nitrogenous base (adenine), a ribose sugar and three phosphate groups (Dunn and Grider, 2020). In the ATP production process, electrons from the electron transport chain escape directly to oxygen, leading to the production of free radicals such as superoxide anion, hydroperoxides, hydroxyl radicals and others (Cui et al., 2012; Martín et al., 2002; Schütt et al., 2012).

These radicals formed during the ATP production process can disrupt the activity of oxidative phosphorylation enzymes, leading to impaired ATP synthesis. (Martín et al., 2002). Disruption in ATP synthesis also adversely affects GSH production. This is because GSH synthesis occurs through a two-step enzymatic process requiring ATP (Lu, 2013). Decreased ATP levels in the oxidative process reduce the effectiveness of antioxidant defense systems that are essential for cellular protection. Unpreventable oxidative attack can further reduce ATP levels and cause a vicious cycle (Lu, 2013).

Mitochondria are considered to be a major target of oxidative attack as well as a source of ROS (López et al., 2009). A large body of literature indicates that mitochondrial functions can be altered by OS (Liang et al., 2007). In a study, it was reported that OS inhibits respiratory chain enzyme complexes and reduces ATP production (Liang et al., 2007). On the other hand, the mitochondrial inner membrane, where ATP production takes place, is rich in polyunsaturated fatty acids, making it susceptible to oxidation (Liang et al., 2007). For example, cardiolipin, an important lipid component of the inner mitochondrial membrane, plays a critical role in the function of mitochondrial proteins such as cytochrome oxidase and its oxidation disrupts mitochondrial activities (Van Remmen and Richardson, 2001).

In the literature, the decrease in ATP levels in OS has been attributed to the increase in its consumption as well as the decrease in its production. Because, repair mechanisms that increase as a result of oxidative damage also require ATP (Wang et al., 2003).

Overall, ATP is essential for the functioning of the cell because it provides the energy for many cellular reactions. For example, ATP provides energy for mechanisms that maintain the correct concentrations of charged particles (i.e. ions such as sodium, potassium, or calcium) in the cell (Nanji & Hiller-Sturmhöfel, 1997). As a result of cellular ATP deficiency, the release of calcium ions from intracellular stores can result in apoptosis. The process of apoptosis is a form of controlled cell death that requires a small amount of ATP (Prauchner, 2017). When ATP levels are further reduced or depleted, apoptosis is replaced by necrosis, which is uncontrolled cell death (Prauchner, 2017). In the literature, it has been reported that both apoptosis and necrosis caused by ATP deficiency are mainly caused by OS and increase in mitochondrial permeability (Prauchner, 2017).

Several studies have been conducted in the past to demonstrate the relationship between OS and cellular ATP levels (Schütt et al., 2012; Agalakova & Gusev, 2012; De Cristóbal et al., 2002). In an *in vitro* study in retinal pigment epithelial cells, Schütt et al. examined the relationship

between decreased ATP synthesis and oxidative damage to intracellular GSH levels, cellular proteins and DNA and concluded that moderately decreased intracellular ATP levels may contribute to oxidative stress damage and dysfunction (Schütt et al., 2012).

Agalakova et al., evaluated ROS accumulation and changes in glutathione and ATP contents in rat erythrocytes in oxidative stress induced by inorganic fluoride. They revealed that ATP concentration showed a dose- and time-dependent decrease in the oxidative process. They pointed out that GSH synthesis and GSH/oxidized glutathione membrane transport are ATP-dependent processes and therefore ATP depletion may be a cause of impaired GSH regeneration in rat erythrocytes (Agalakova & Gusev, 2012).

In another study, it was shown that acute immobilisation stress was accompanied by an increase in lipid peroxidation and a decrease in reduced GSH in the rat brain and a decrease in brain ATP levels (De Cristóbal et al., 2002).

Some researchers have also examined how OS responds to exogenous ATP treatment (Aldemir et al., 2020; Dagel et al., 2024). Aldemir et al. reported that ATP administration blocked the oxidative toxicity of sunitinib in sunitinib-induced cardiotoxicity (Aldemir et al., 2020). In another recent study, it was reported that ATP treatment prevented the increase in oxidant levels and decrease in antioxidants due to 5-fluorouracil treatment and protected renal tissue from oxidative damage (Dagel et al., 2024).

Although the literature is rich in studies on ATP, the use of ATP therapy in OS is very limited and mostly includes preclinical studies (Schütt et al. 2012; De Cristóbal et al., 2002). The results of preclinical studies are encouraging for the trial of ATP application for clinical studies (Aldemir et al., 2020; Dagel et al., 2024).

## Conclusion

OS refers to the deterioration of the balance between oxidants and antioxidants in favor of oxidants, and this may lead to disruptions in redox signaling and control and/or damage at the molecular level. The main source of ROS, which is the source of oxidative stress, is mitochondria and at the same time mitochondria is one of the main organelles targeted against oxidative attack. Therefore, damage to mitochondria leads to disruption of oxidative phosphorylation and ATP synthesis. On the other hand, the necessity of ATP synthesis for antioxidant defence makes tissues vulnerable in case of deficiency. In this case, it is likely that tissues exposed to OS also have problems in ATP production and the decrease in ATP synthesis further increases oxidative damage. In order to protect against OS, which has been found to play a role in the pathogenesis of many diseases, new treatment strategies to maintain ATP levels and exogenous ATP therapy may be an important field of research.

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# The Therapeutic Effect of Decompression Surgery on Motor and Cognitive Function Losses as a Result of Haemorrhagic Stroke in a Hypertensive Patient: A Case Study

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## ABSTRACT

Stroke is a serious cerebrovascular disease that can cause disability and death if not diagnosed and treated early. Stroke is the leading cause of death among neurological diseases. In this case study, we describe the therapeutic history of decompression surgery in a 72-year-old patient with motor and cognitive function deficits after acute ischaemic stroke.

**Keywords:** Haemorrhagic Stroke, Hypertension, Physiotherapy

## Introduction

Cerebral blood flow is the most important part of the brain's nutrition and cerebral ischaemia occurs as a result of a decrease in cerebral blood flow in a selected brain region or in the whole brain (Demirdogen, Ozdemir, Akcay, Iyigun, 2023). When the cerebral artery supplying blood to the brain is blocked or ruptured, the blood flow in the central region of the area supplied by the artery falls below the critical level and the blood flow in the areas immediately surrounding the centre is insufficient to maintain the function and vitality of the neurons (Demirdogen et al., 2023). Ischaemic stroke accounts for 87%, intracerebral haemorrhage for 10% and subarachnoid haemorrhage for 3% of all strokes in the World (Benjamin et al., 2018). It has been found that the prevalence of ischaemic stroke is 176/100,000 and haemorrhagic stroke is 90/100,00 in people under 65 years of age worldwide, while the prevalence of ischaemic stroke is 300/100,000 and the prevalence of haemorrhagic stroke is 116/100,000 in people over 65 years of age (Krishnamurthi et al., 2015). Significant impairments in both motor and cognitive functions occur after stroke (Akçay et al., 2024). Although stroke has a high incidence, there is no effective drug treatment yet. Hypertension, diet, alcohol, age, diabetes and gender are factors affecting stroke. In this case report, a 72-year-old woman with hypertension suddenly developed right-sided weakness and loss of consciousness. Computed tomography (CT) revealed haemorrhage in the left middle cerebral artery (MCA). Brain magnetic resonance imaging was performed for differential diagnosis and haemorrhagic infarction was detected. Decompression surgery was carried out.

## Case Presentation

72-year-old female patient. The patient with a known diagnosis of hypertension suddenly developed right-sided weakness and loss of consciousness at 07:30. The patient was transferred by ambulance and admitted to the emergency department of the hospital at 08:30. Brain CT and Diffusion MR imaging were performed at 08:45. Brain magnetic resonance imaging was performed for differential diagnosis after a haemorrhage was detected in the area corresponding to the left MCA area on CT. Sinus vein thrombosis was not detected. Diffusion MRI revealed a haemorrhage area with diffusion restrictions in the temporoparietofrontal region. It was evaluated as haemorrhagic infarction. Neurological Examination: consciousness is somnolent. Motor deficit: right hemiplegia. Broka aphasia is present. The patient had high blood pressure and blood pressure was kept around 160/85.

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Routine haemogram and biochemistry tests were within normal range. The patient developed anisocoria during follow-up and since an increase in shifting was detected in the control CT scan, the patient was consulted to neurosurgery. Decompression surgery was performed.

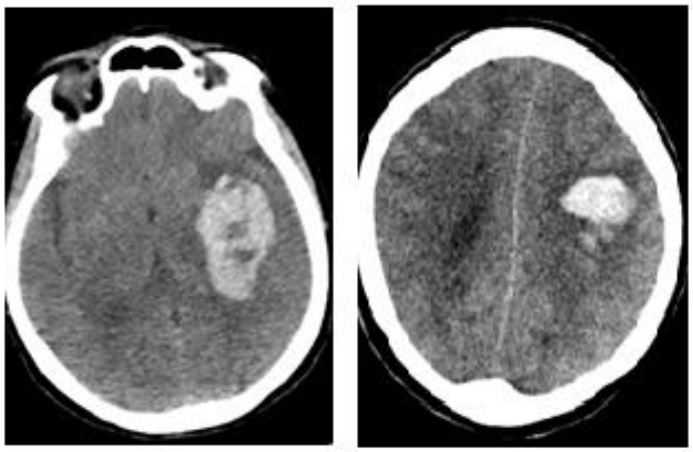

**Figure 1.** CT imaging on first arrival to the emergency department. Heterogeneous intracerebral haemorrhage area with oedematous hypodensity in the left temporoparietofrontal area and periphery, causing 11 mm right shift

The patient was administered 20% Mannitol 4x125 cc intravenously. It was planned to decrease and stop according to clinical and radiological follow-up. However, because the patient's consciousness continued somnolent and cytotoxic oedema and shifting continued on control CT, dexamethasone 8 mg 4x1/2 was added to the treatment and it was planned to be gradually decreased and stopped. Because the general condition of the patient did not improve despite the treatment, consciousness did not return, respiratory pattern deteriorated and anisocoria developed, control brain tomography was performed and the patient was consulted to the neurosurgery department. Decompression surgery was deemed appropriate because the bleeding area was large, oedema continued to increase despite anti-oedema medical treatments, and shifting increased.

Decompression surgery was performed. During the postoperative follow-up, the patient regained consciousness. Anisocoria improved. Right lower extremity strength was 2/5, while no change was found in right upper extremity strength. Escitalopram was added to the patient whose consciousness and awareness increased, because of crying fits. It was observed that crying spells decreased. Blood and urine cultures were taken because the patient had fever several times during the follow-up period. Escherichia coli (E.coli) was found in urine culture and Pseudomonas aeruginosa was found in blood culture. Vancomycin and meronem were started. According to clinical and laboratory findings, the treatment period was completed to 14 days and ended.

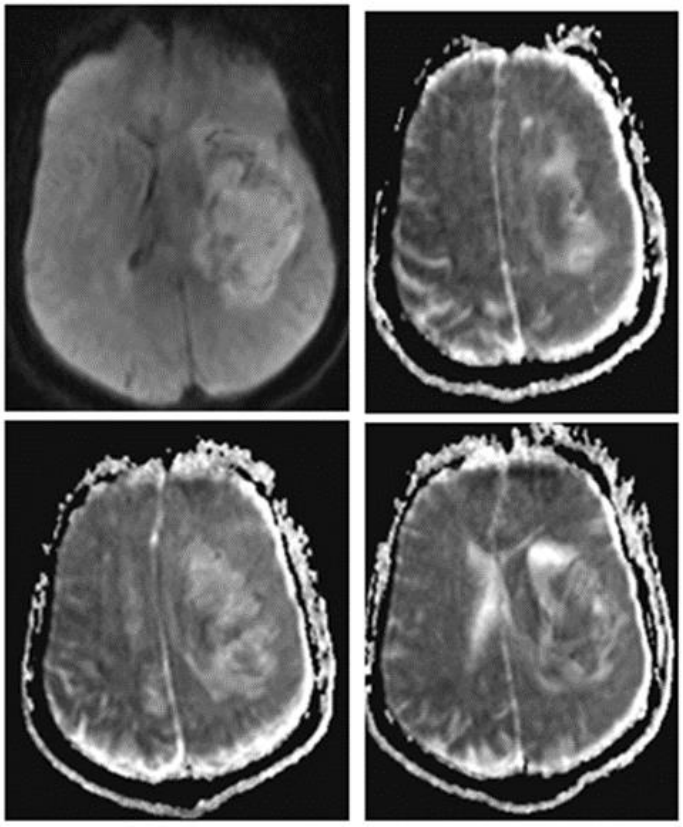

**Figure 2.** Emergency first arrival diffusion mrg (b1000 and ADC sequence). A haemorrhage area in the temporoparietofrontal region of the left cerebral hemisphere was detected with oedema compressing the left lateral ventricle, including areas of diffusion restriction

## Discussion

Stroke is the third leading cause of death and disability in the world after cardiovascular and cancer diseases (Akçay, 2021). Approximately 87% of stroke cases are ischaemic stroke and 13% are haemorrhagic stroke. Ischaemic stroke occurs as a result of rupture or blockage of the vessels feeding the brain (Akçay, 2021). Worldwide, the risk of stroke increases with increasing age and especially after the age of 55 years, the risk of stroke increases 2-fold in each decade (Boehme, Esenwa, Elkind, 2017). Since the elderly population is increasing in developed and developing countries, it is estimated that there will be a rapid increase in stroke cases in the future, which will cause health and economic problems (Akçay, 2021). As a result, prevention of stroke and implementation of effective treatments are also important for our country. Antiaggregant, anticoagulant, thrombolytic, antioedema

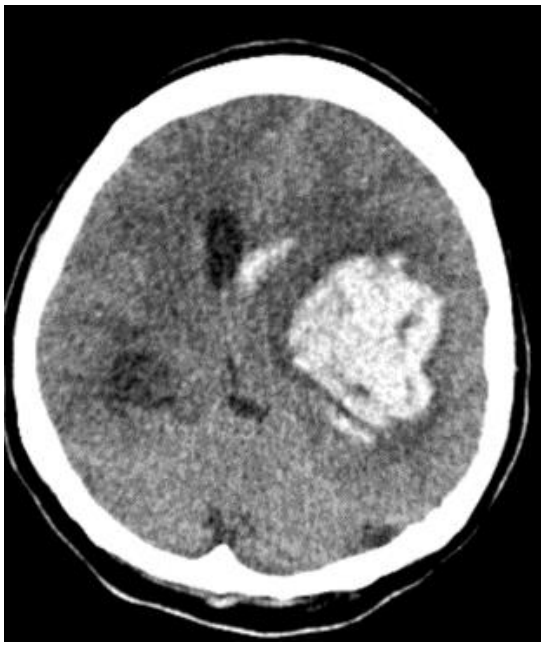

**Figure 3.** Image after anti-edema treatment. Intracerebral haemorrhage area with heterogeneous character, oedematous hypodensity in the left temporoparietofrontal area and periphery, causing 11 mm right shift persists.

and neuroprotective agents are widely used in stroke treatment (Akçay, 2021).

Haemorrhage may occur spontaneously after cerebral infarction or due to antithrombotic or thrombolytic therapy (Sacco et al., 2013). Haemorrhage after infarction may vary from minor petechial haemorrhages to major haemorrhages; these conditions are called haemorrhagic infarction, haemorrhagic transformation and parenchymal haemorrhage. Haemorrhagic transformation type 1 is a condition in which petechiae form at the edges of the infarct; type 2 is a condition in which petechiae are distributed within the infarct area without covering an additional area. In parenchymal haemorrhage, a mass effect occurs with focal blood collection. Parenchymal haemorrhage type 1 refers to haemorrhages that cover up to 30% of the infarcted area and have a mild space-occupying characteristic; type 2 refers to haemorrhages that cover more than 30% of the infarcted area and/or create a significant space-occupying effect. These conditions need to be treated as intracerebral haemorrhage and are considered in the intracerebral haemorrhage group. Our case was in the type 2 group with haemorrhage covering more than 30% of the infarcted area and/or causing a significant space-occupying effect.

Progressive cerebral brain oedema occurs in 10% of ischaemic strokes and clinical deterioration may occur rapidly within 2-5 days. Early decompression surgery (DS) is important in dealing with increased intracranial pressure and cerebral oedema (Ronchetti et al., 2014). Although studies report that DS increases disability in individuals, DS is important in terms of reducing mortality. In this case, DS is one of the treatment options. However, there is no clear consensus on patient selection and timing of DS (Beez et al., 2019). Considering the increasing number of patients diagnosed with stroke at a young age, DS is more important (Zweckberger et al., 2014). Although age is one of the most important areas of interest for the DS decision, it is controversial for which age it will be more beneficial (Powers et al., 2019). Agarwalla et al (2014) reported that DS reduces mortality and disability in younger patients (Agarwalla et al., 2014). Although different studies have reported a poor prognosis in the group over 55 years of age (compared to medical treatment) despite increased survival with DS, similar to the results of this study, there are also studies reporting that age is not a factor affecting mortality (Daou et al., 2016; Jüttler et al., 2014; Zweckberger et al., 2014). Therefore, age may not be an

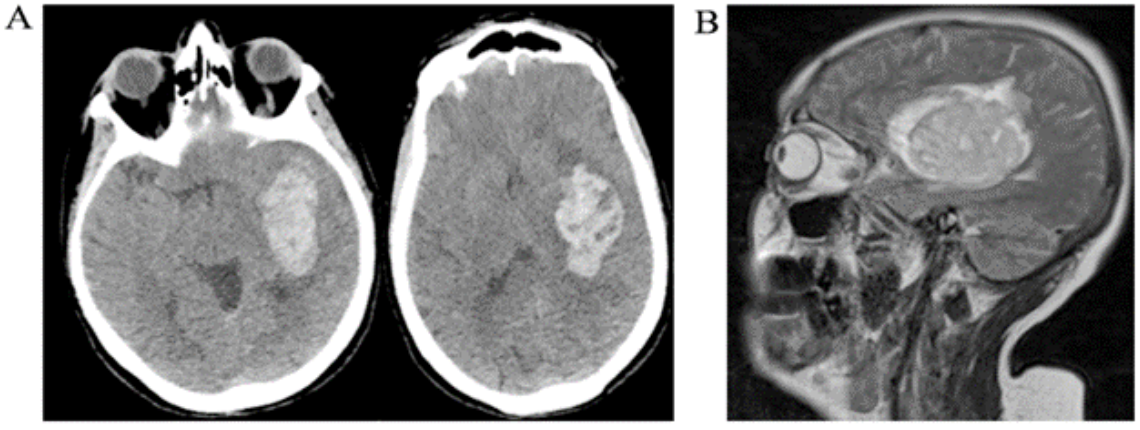


**Figure 4.** A) Pre-operative CT: left lateral ventricle compression of left hemisphere bleeding area-right lateral ventricle compression with shifting effect, B) Pre-operative Sagittal Section MR: left lateral hemisphere bleeding area with large oedema around it.

indicator for patient selection (Daou et al., 2016). The optimal DS time for MCA infarcts is unknown. In stroke patients, decompression within 24 hours or before clinical signs of herniation may improve mortality and functional outcomes (Shah et al., 2016). Neurological deterioration occurs within 5 days of stroke onset, with the highest mortality rate due to transtentorial herniation and subsequent brain death occurring within 3 days. The mortality rate due to malignant MCA infarction is around 80% without surgical intervention (Agarwalla et al., 2014). Agarwalla et al. reported that oedema-related deterioration in MCA infarcts is most common in the 48-hour time interval. (Agarwalla et al., 2014). In this study, 9 patients (52.9%) were processed in the first 24 hours, 5 patients (29.4%) in the first 48 hours, and 3 patients (17.6%) in 72 hours or later. Although there are studies reporting that stroke patients should be admitted to DS within the first 24 hours from the onset of symptoms, the widely accepted view is that the critical period for DS is the first 48 hours (Shah et al., 2016; Wijdicks et al., 2014).

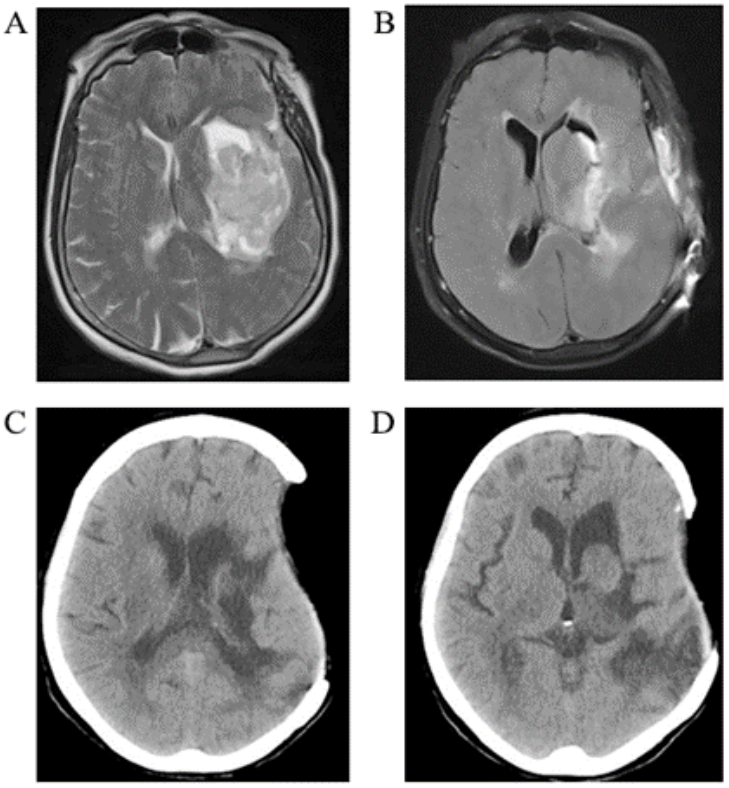


**Figure 5.** A) Pre-decompression operative MR (T2 Sequence): left lateral ventricular compression due to haemorrhage in the left cerebral hemisphere and right lateral ventricular compression due to shunting; B) Post-decompression operative 24th hour MR (T2 Fair): decrease in shunting and decrease in right lateral ventricular compression 24 hours after the operation C) Post-decompression operative 6th month CT, D) Post-decompression operative 6th month CT: 6 months after the operation, marked improvement of shifting and removal of compression on both ventricles

In this case report, a 72-year-old hypertensive female patient suddenly developed right-sided weakness and loss of consciousness and underwent decompression surgery for haemorrhagic infarction in the left MCA. After decompression surgery, the patient's shifting decreased. The patient has been treated in the palliative service for more than 1 year, and as a result of drug therapy and physiotherapy, the patient's last condition has improved motor function in the left arm and leg, but loss of function continues on the right side. Although cognitive function is partially restored, the patient is aphasic.

## Conclusion

In conclusion, the ischaemic stroke caused by haemorrhagic infarction in a hypertensive patient resulted in permanent damage to motor and cognitive functions. After decompression surgery, the patient was intubated for a long period of time and then extubated and continued with medication and partially recovered motor and cognitive functions. Motor function continues to be reinforced with physiotherapy.

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**Peer-review**: Externally peer-reviewed.
**Author Contributions:** Concept - G.A., D.K.; Design- G.A., D.K.; Supervision- G.A., D.K.; Resources- G.A., D.K.; Data Collection and/or Processing- G.A., D.K.; Analysis and/or Interpretation- G.A., D.K.; Literature Search- G.A., D.K.; Writing Manuscript- G.A., D.K.; Critical Review- G.A., D.K..
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