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Comparative Nephroprotective Effects of Silymarin, N-Acetylcysteine, and Thymoquinone Against Carbon Tetrachloride-Induced Nephrotoxicity in Rats

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Abstract

Background: Many pharmacological agents may lead to kidney damage. Preventing nephrotoxicity reduces the risk of morbidity and mortality, as well as decreasing hospitalization costs.

Objectives: In this study, we investigated the comparative nephroprotective effects of silymarin, N-acetylcysteine (NAC), and thymoquinone (TQ) in animal models (rats) in which we induced nephrotoxicity using carbon tetrachloride (CCl4).

Methods: This animal experimental study was conducted at the experimental animals center of Yuzuncu Yil University, Turkey, in 2015. Thirty-eight adult male Wistar rats were used in this study. We defined five experimental groups and treated them for four weeks. The first group (n = 8) was given no medicine. The second group (n = 8) was given only CCl4 (1.5 ml/kg, intraperitoneally (IP), in olive oil, twice a week). The third group (n = 6) was given TQ (10 mg/kg, IP, in dimethyl sulfoxide (DMSO), daily) and CCl4 (1.5 mL/kg). The fourth group (n = 8) was given silymarin (100 mg/kg, IP, in DMSO, daily) and CCl4 (1.5 mL/kg), while the fifth group (n = 8) was given NAC (10 mg/kg, IP, daily) and CCl4 (1.5 mL/kg). The kidneys of all the rats in every group were evaluated histologically using light microscopic methods at the end of the fourth week. A grading scheme was used to score the histological alterations related to tubular injury: absent (-), mild (+), moderate (++), severe (+++), and quite severe (++++).

Results: In terms of the mean values of tubular damage, the first group had a mean of 0.0, the second group had 3.88 $\pm$ 0.35, the third group had 1.00 $\pm$ 0.89, the fourth group had 2.13 $\pm$ 1.13, and the fifth group had 2.75 $\pm$ 1.04. The results showed that, histopathologically, CCl4 had quite a severe toxic effect on the tubules when compared to the control group, although the glomeruli were intact. Silymarin, TQ, and NAC all showed statistically significant nephroprotective effects ($P < 0.01$). However, of the three, TQ was the most powerful nephroprotective agent ($P < 0.01$).

Conclusions: In conclusion, we suggest that TQ may be used as a prophylactic agent against nephrotoxicity, especially in instances of tubular injury. However, human-based studies are still needed.

Keywords: Kidney, Nephrotoxicity, Nephroprotective, Silymarin, N-Acetylcysteine, Thymoquinone

1. Background

Carbon tetrachloride (CCl4) is a well-known agent that is commonly used in the dry-cleaning industry. It has been proven to have a highly hepatotoxic effect, as well as inducing nephrotoxicity. CCl4 can lead to acute tubular necrosis in the kidney and damage to the liver, which leads to cirrhosis (1, 2). Its harmful effect on the liver and kidney occurs due to the CCl4 metabolites, toxic trichloromethyl, and trichloromethylperoxy radicals inherent in the cytochrome P450 system (3).

N-acetylcysteine (NAC) is described as an antioxidant that prevents oxidative injury to issues due to directly binding hydroxyl radicals and increasing glutathione production (4). Due to its optimal thiol redox state, NAC balances and optimizes cells against oxidative stress and inflammation (5).

Silymarin is a flavonoid complex that is extracted from the seeds of *Silybum marianum*. It is a mixture of silibinin, isosilybinin, silychristine, and silydianine. Silymarin produces a hepatoprotective effect due to its radical scavenger effects (6). Due to being an antioxidant, silymarin stabilizes cell membranes, regulates the intracellular content of reduced glutathione, and chelates metal ions (iron and copper) (7, 8).

Thymoquinone (TQ) is a flavonoid bioactive con-

stituent that is extracted from the seeds of Nigella sativa, which is commonly known as cumin and used in folk medicine to cure several conditions (9). TQ has a protective effect on some tissues, such as those in the liver (10) and heart (11). Recent studies have shown that TQ also has a nephroprotective effect, especially against oxidative stress and inflammation (12).

There are many pharmacological agents that can cause kidney damage. Preventing nephrotoxicity through the use of cheap and effective drugs decreases hospitalization costs, as well as lowering rates of morbidity and mortality. As mentioned above, silymarin, NAC, and TQ have all been reported to be nephroprotective. However, there has been no previous comparative study concerning the nephroprotective effects of these three agents. Hence, this study was conducted in order to identify more effective and cheaper nephroprotective drugs, since we know that many such agents are characterized by high costs, especially silymarin, which is used in instances of serious hepatotoxicity such as mushroom poisoning.

2. Objectives

In this study, we observed and compared the nephroprotective effects of silymarin, NAC, and TQ in rats suffering from renal injuries induced by CCl4.

3. Methods

3.1. Animals and Study Design

The experimental protocol was approved by the Local Ethics Committee for Animal Experiments of Yuzuncu Yil University, Van, Turkey (approval number 2015/14). Thirty-eight adult male Wistar albino rats weighing 300 - 350 g were used in this study. The animals were bred and kept in standard conditions (light cycle: 12 hours light/12 hours dark; temperature: 22°C; humidity: 60 +/- 5%) at the experimental animals center of Yuzuncu Yil University. During the study, the rats were fed pellets and given water; they were not given a special diet. The rats were divided into five groups. Explanations of the groups and the administration of the experimental drugs are presented in Tables 1 and 2, respectively. At the end of the fourth week, the rats were euthanized with general anesthesia. The rats were anesthetized with xylazine (10 mg/kg) and ketamine (90 mg/kg), which were administered intraperitoneally. The kidneys of all the rats in every group were then evaluated histologically using light microscopic methods.

3.2. Chemicals

The carbon tetrachloride (99.5%, Ak Kimya, Istanbul, Turkey), silymarin (SIGMA, Missouri, USA), thymoquinone (ALDRICH, Missouri, USA), and N-acetylcysteine (Asist 100 mg/mL ampoule, Husnu Arsan, Istanbul, Turkey) were obtained from the respective manufacturers. The silymarin and thymoquinone were dissolved in dimethyl sulfoxide (DMSO, 20%, Merck, Hohenbrunn, Germany), while the CCl4 was dissolved in olive oil at a ratio of 1: 2.

3.3. Serum Biochemical Analysis

All the animals were euthanized at the end of the fourth week. Blood samples were collected from the animals in order to analyze their biochemical parameters. The blood samples were obtained using BD Vacutainer Systems' vacuum biochemistry tubes. The samples were centrifuged at 3500 rpm for 10 minutes after being held for 30 minutes at room temperature. The serum urea concentration was tested using a urease method, while the serum creatinine concentration was tested according to the Jaffe method in an Abbott ARCHITECT c8000 automatic spectrophotometric analyzer (Abbott Diagnostics Division, USA) using commercially available kits. The blood urea nitrogen (BUN) was calculated as urea/2.14. The serum urea concentration was determined using an enzymatic method, and the serum creatinine concentration was estimated using the Jaffe method. All the serum samples were measured using a Cobas C 501 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) with commercially available kits. All the equipment was calibrated prior to use.

3.4. Histopathological Study

The kidney tissues were fixed with 10% formalin solution, made into paraffin blocks, and then 4 mm sections were obtained. Histological staining was performed using hematoxylin and eosin (H&E), periodic acid shift (PAS), and Masson's trichrome stain. Two pathologists performed the histopathological study as a double-blind study. The sections were assessed using light microscopy. All the sections were assessed to determine the degree of glomerular and tubular injury, vascular congestion, interstitial mononuclear inflammatory cell infiltration, and fibrosis. The following grading scheme was used to score the histological alterations that resulted in tubular injury: absent (-), mild (+, patchy damage to tubules), moderate (++, less than 25% damage to tubules), severe (+++, 25% - 50% damage to tubules), and quite severe (++++, more than 50% damage to tubules).

Table 1. Explanation of the Groups

	Explanation
1	Control group (n = 8); did not receive any medicine
2	CCl4 group (n = 8); given only CCl4
3	TQ group (n = 6); treated with TQ and CCl4
4	Silymarin group (n = 8); treated with silymarin and CCl4
5	NAC group (n = 8); treated with NAC and CCl4

Abbreviations: CCl4, carbon tetrachloride; TQ, thymoquinone; NAC, N-acetylcysteine.

Table 2. Administration of the Experimental Drugs

	Administration
CCl4	Dissolved in a 1: 2 mixture with olive oil, 1.5 mL/kg dose, IP, twice a week for 4 weeks
TQ	Dissolved in DMSO, 10 mg/kg daily, IP, for 4 weeks
Silymarin	Dissolved in DMSO, 100 mg/kg daily, IP, for 4 weeks
NAC	100 mg/kg, IP, daily for 4 weeks

Abbreviations: CCl4, carbon tetrachloride; DMSO, dimethyl sulfoxide; IP, intraperitoneally; TQ, thymoquinone; NAC, N-acetylcysteine.

3.5. Statistical Analysis

The data were analyzed using SPSS version 20.0 statistical software. Differences between the groups were measured using the Kruskal-Wallis H test. All values are given as mean ± SD for the animals in each group. A value of $P < 0.05$ was considered to be statistically significant. A power calculation was performed for the study. Important data were for power is 80%, the Type 1 error was 5%, and the sample size was six rats.

4. Results

Comparisons of the histopathologic findings in all the groups are presented in Table 3. CCl4 exhibited remarkable potential for tubular damage. When the nephroprotective effects of NAC, TQ, and silymarin against tubular damage were compared, TQ showed the most powerful effect ($P < 0.05$). Silymarin and NAC also exhibited a protective effect ($P < 0.05$); although it was lower than that of TQ. No statistical difference was found between the silymarin and NAC groups. There was also no statistically significance difference in terms of kidney weight, interstitial mononuclear inflammation, or interstitial fibrosis between any of the five groups. The levels of serum BUN, urea, and creatinine in all the groups are presented in Table 4. All values are given as mean ± SD for the animals in each group. The data shows that there was no statistically significance difference in terms of the serum creatinine, BUN, or urea levels between any of the groups ($P > 0.05$).

4.1. Histological Examination

The histopathological examinations revealed changes of varying severity, including none (control group; Figure 1C), mild (TQ + CCl4 group; Figure 1F), moderate (silymarin + CCl4 group; Figure 1E; NAC + CCl4 group; Figure 1D), and quite severe (CCl4 group; Figure 1A and 1B). In the CCl4-exposed kidneys, the histological examinations revealed decreased brush borders, loss of brush border, bare basement membranes, karyolysis, tubular dilatation, tubular necrotic areas, hydropic degeneration in tubular epithelium cells (especially in the proximal tubules), intraluminal cast (especially in the distal tubules), and poured epithelium cells in tubule lumens (Figure 1). Medullar congestion was also observed. However, the glomeruli were intact, and there was no evidence of fibrosis or interstitial inflammation. Moreover, the control group had intact kidney structures, and no pathological signs were evident during light microscope investigations using periodic acid shift (PAS), Masson's trichrome stain, and hematoxylin and eosin stains.The use of silymarin, NAC, and TQ significantly reduced the kidney injuries and protected against CCl4-induced tubular damage. However, TQ had the most powerful nephroprotective effect of the three agents. Silymarin and NAC also had some nephroprotective effects, although they were not as effective as TQ.

Table 3. Comparison of the Histopathologic Findings for the Five Groups [a]

	Mean	St. Dev.	Median	IQR	P
Kidney weight					0.662
Control	1.23	0.16	1.25	0.28	
CCl4	1.20	0.14	1.27	0.14	
TQ + CCl4	1.32	0.38	1.20	0.13	
Silymarin + CCl4	1.29	0.17	1.28	0.16	
NAC + CCl4	1.15	0.17	1.18	0.28	
Tubular damage					0.001
Control	0^{A}	0	0	1	
CCl4	3.88^{B}	0.35	4.00	1	
TQ + CCl4	1.00^{C}	0.89	1.00	1	
Silymarin + CCl4	2.13^{D}	1.13	2.00	1	
NAC + CCl4	2.75^{D}	1.04	3.00	2	
Interstitial mononuclear inflammation					0.841
Control	0.13	0.35	0	0	
CCl4	0.13	0.35	0	0	
TQ + CCl4	0.17	0.41	0	0	
Silymarin + CCl4	0.25	0.71	0	0	
NAC + CCl4	0	0	0	0	
Interstitial fibrosis					0.577
Control	0	0	0	0	
CCl4	0.13	0.35	0	0	
TQ + CCl4	0	0	0	0	
Silymarin + CCl4	0.25	0.71	0	0	
NAC + CCl4	0	0	0	0	

Abbreviations: CCl4, carbon tetrachloride; TQ, thymoquinone; NAC, N-acetylcysteine; St. Dev., standard deviation

[a] There was a statistically significance difference between all the groups (A, B, C, D) (except between the Silymarin + CCl4D and NAC + CCl4D groups) in terms of tubular damage ($P < 0.05$). However, there was no statistically significance difference between the groups in terms of kidney weight, interstitial mononuclear inflammation, or interstitial fibrosis ($P > 0.05$).

5. Discussion

CCl4 seems to be a potent agent leading to tubular damage. However, although tubular damage was observed, the glomeruli were intact. Additionally, there was no interstitial mononuclear inflammation or interstitial fibrosis. The findings of previous studies differ in this regard. In Ozturk et al.'s study, CCl4 had the potential to result in serious cortical damage and focal glomerular necrosis. In this study, tubular dilation and the lining of flattened epithelial cells were found, apoptosis occurred on the basal membrane and necrotic epithelial cells, and CCl4 was given daily in 1 mL/kg doses for 11 days. In addition to the tubular damage, glomerular damage and necrotic glomerular areas were found in Ozturk et al.'s study (13).

In another previous study, the effect of CCl4 on the medulla was found to be limited. Glomerular involvement is seen in different forms of degradation. Some glomeruli show Bowman's capsule dilatation, glomerular atrophy, capillary loop congestion, or adhesion of the Bowman's capsule layers. There is also tubular dilatation, and the vacuolated epithelia have a foamy appearance. Inflammatory cell infiltration occurs following CCl4 exposure. Further, on the cortico-medullary border, there is mild mononuclear inflammatory cell infiltration, and there appear to be connective tissue cells near the inflammation. All these findings were observed in the renal cortex and subcortical areas (14). In our study, there was no significant glomerular damage, although tubular injuries were obvious.

Studies have shown that TQ clears up free radicals and

Table 4. Comparison of the Serum Urea, Creatinine, and BUN Test Results for the Five Groups[a]

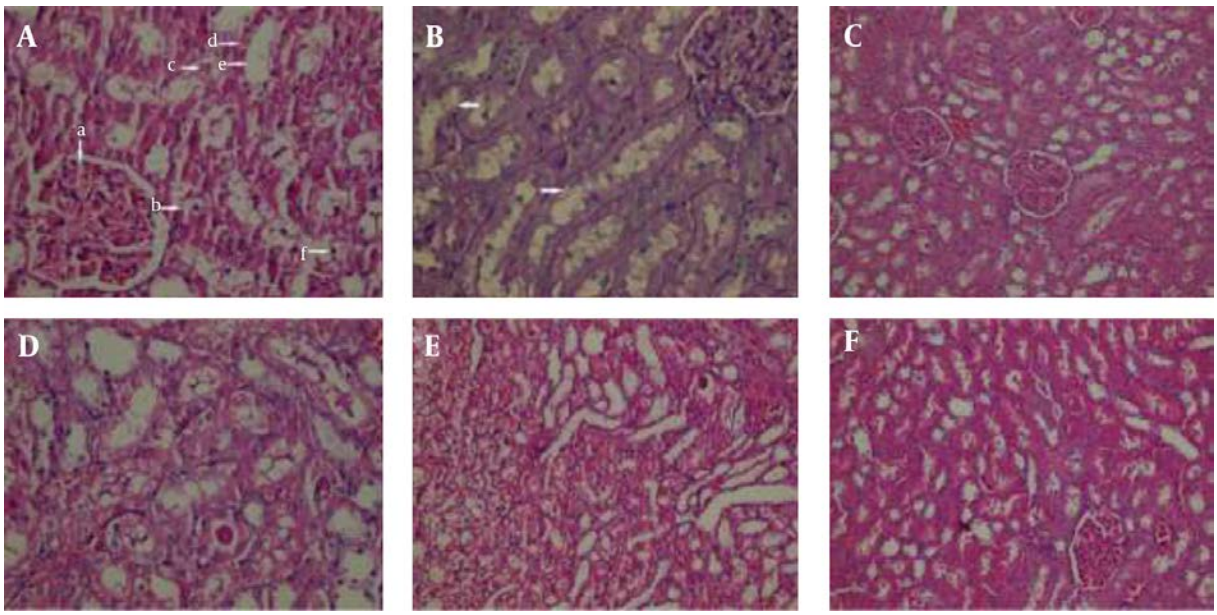
	Mean	St. Dev.	Median	IQR	P
Serum creatinine levels, mg/dL					0.59
Control	0.49	0.02	0.49	0.03	
CCl4	0.51	0.11	0.46	0.09	
TQ + CCl4	0.45	0.02	0.45	0.02	
Silymarin + CCl4	0.46	0.06	0.46	0.05	
NAC + CCl4	0.50	0.04	0.49	0.06	
Serum urea levels, mg/dL					0.65
Control	53.50	5.21	51	8	
CCl4	50.87	13.00	46	13	
TQ + CCl4	44.33	5.32	44	3	
Silymarin + CCl4	51.25	3.45	51	4	
NAC + CCl4	49.33	6.62	49	13	
BUN levels, mg/dL					0.65
Control	24.65	2.40	23.50	3.69	
CCl4	23.45	5.99	21.20	5.99	
TQ + CCl4	20.43	2.45	20.05	1.39	
Silymarin + CCl4	23.62	1.59	23.27	1.84	
NAC + CCl4	22.73	3.05	22.35	5.99	

Abbreviations: CCl4, carbon tetrachloride; TQ, thymoquinone; NAC, N-acetylcysteine; St. Dev., standard deviation; BUN, blood urea nitrogen.
[a]There was no statistically significance difference between any of the groups in terms of the serum creatinine, urea, and BUN levels (P > 0.05).

inhibits lipid peroxidation, as well as having a therapeutic effect on some in vitro and in vivo models such as cancer- (15), *E. coli*-induced pyelonephritis- (16), vancomycin- (17), cadmium- (18), arsenic- (19), cyclosporine A- (20), cisplatin- (21), gentamicin- (22), and methotrexate-induced (23) nephrotoxicity in rats. In many studies, thymoquinone has been shown to be nephroprotective in other diseases. For example, a Pakistan-based study (24) revealed that TQ is effective during rheumatoid arthritis treatment in terms of reducing the inflammation and protecting the kidney. Its nephroprotective effect seems to be better than that of methotrexate. Additionally, Kanter (25) showed that treatment with TQ prevents streptozotocin-induced diabetic nephropathy. It normalizes the glomerular and capsular size, as well as reducing the tubular basement membrane thickness. Further, TQ ameliorates high levels of mesangial matrices, tubular dilation, and renal function. Another prior study revealed that TQ ameliorates the renal proliferative response due to mercury exposure in rats. TQ decreases histological damage such as renal cell apoptosis and proliferative reactions (26). While many studies have suggested that TQ is a nephroprotective agent, in the present study we further suggest that it has a higher nephroprotective effect than either NAC or silymarin.

In the literature, NAC therapy has been proven to be beneficial for many clinical conditions, including kidney, intestinal, liver, rheumatologic, pulmonary, and infectious diseases (27-29). Additionally, some studies have suggested that, since NAC protects cells from oxygen-derived free radicals, it also improves renal vasodilatation and hence protects renal functions against kidney injury due to radiocontrast agents (29). Many studies have revealed that NAC has a nephroprotective effect against different nephrotoxic agents. NAC is useful for relieving aspartame-induced oxidative stress (30), contrast-induced nephropathy (31), amphotericin B-induced acute interstitial nephritis (32), and carbosulfan-induced oxidative damage (33) in humans. However, these studies did not include histopathologic surveys. Finamor et al. (30) analyzed reactive substances in rat kidneys in supernatants after the centrifugation of whole kidneys. As NAC is an antioxidant, it protects the kidney cells from oxidative injuries. The literature is concordant with the findings of our study, and we conclude that NAC is useful in clinical conditions that occur with oxidative stress. In our study, the histopathological findings for the NAC+ CCl4 group are more favorable than

Figure 1. Histopathologic Findings Using Light Microscopy



A, CCl4 group; almost normal glomeruli structure (a), CCl4-induced nephrotoxicity such as hydropic degeneration in the tubule epithelium (b), intraluminal cast (c), karyolysis (d), tubular dilatation (e), and epithelium cells in the tubule lumens (f) can be seen (hematoxylin-eosin, ×40); B, CCl4 group; the loss of the brush border in the tubular epithelium cells can be seen (uncolored, arrow) (periodic acid-Schiff, ×40); C, Control group; normal glomeruli and tubules (hematoxylin-eosin, ×20); D, NAC + CCl4 group; the nephrotoxic effect decreases, as seen in the picture (hematoxylin-eosin, ×40); E, Silymarin + CCl4 group; the nephrotoxic effect decreases, as seen in the picture (hematoxylin-eosin, ×20); F, Thymoquinone + CCl4 group; TQ seems to substantially ameliorate the nephrotoxic effect of CCl4 and hence an almost normal tubular structure is seen (hematoxylin-eosin, ×20).

those for the CCl4 group. Kidney weight did not change with the use of NAC. Abdelrahman et al. (34) found that cisplatin causes tubular injuries, although NAC administration protects the kidneys from cisplatin toxicity. The administration of NAC in rat kidneys results in almost intact morphology and histology. Additionally, cisplatin generally increases the plasma, urea, and creatinine levels, but if given with NAC, this alteration does not occur (34). In our study, the serum creatinine and urea levels did not differ among the groups, although NAC was found to protect against tubular injury.

Silymarin has beneficial effects on illnesses affecting different organs, and it could be useful for treating diabetes and a wide range of cancers. A number of studies have suggested that silymarin has anti-fibrotic, anti-lipid peroxidative, anti-inflammatory, immune-modulating, dose-dependent anti-apoptotic, and antioxidant effects (35). It also induces RNA and protein synthesis so that regeneration and reparation increase following renal and liver injury (36). Silymarin has been found to have significant kidney-saving effects in recent research studying doxorubicin- (37), arsenic- (38), manganese- (39), ischemia- (40), reperfusion- (41), and radiation-induced (42) nephrotoxicity in rats. Bektur et al. (43) indicated that an overdose of acetaminophen results in serum BUN and creatinine elevation, as well as liver injury in mice. In the kidney sections, dilation of Bowman's capsular space and glomerular capillaries, pale-staining of tubule epithelium, cell infiltration, and apoptosis occurred. Following treatment with silymarin one hour after the administration of acetaminophen, the histological defects and elevated serum BUN and creatinine levels were all ameliorated (43). In another study, methotrexate was used to induce renal toxicity in rats. In the kidney sections, dilation in Bowman's capsular space, inflammatory cell infiltration, and glomerular and peritubular vascular congestion were found. Additionally, apoptosis was increased. Silymarin treatment led to significant histopathologic improvements in the kidney sections (44). The literature therefore supports our study's finding that silymarin is nephroprotective.

None of the aforementioned prior studies indicated which of the three agents (silymarin, NAC, and TQ) is the most effective. A comparison of the nephroprotective effects of silymarin, NAC, and TQ has not previously been attempted. Hence, the present study is unique. In this study, it was suggested that TQ may be used as a prophylactic agent against nephrotoxicity, especially in cases of tubular

injury. However, a limitation of our study is the low sample size, and human-based studies are still needed.

In conclusion, many studies have found TQ, NAC, and silymarin to be nephroprotective agents. In the present study, we compared the effects of the three agents on CCL4-induced tubule injury. The histopathologic sections showed that TQ seems to have the best nephroprotective effect on tubule injury of the three agents. TQ is an extract of cumin that is cheap, easy to obtain, and used worldwide. We could not find any case of cumin toxicity in the literature. TQ is likely to be effective as a prophylactic medicine against nephrotoxicity, especially in clinical conditions such as tubulopathy. We therefore suggest that TQ may be a useful prophylactic agent against nephrotoxicity, especially in cases of tubular injury, although human-based studies are still needed.

Footnote

Authors' Contribution: Lokman Ustyol made substantial contributions to the conception of the study, the bibliography, and drafting the manuscript. Kaan Demirören was involved in the statistical analysis and interpretation of the data. Ibrahim Kandemir was involved in the acquisition of data and bibliographic research. Remzi Erten conducted the pathologic evaluation. Kezban Bulan and Sultan Kaba were involved in critically revising the manuscript for important intellectual content. Nihat Demir and Mehmet Turan Basunlu were involved in the conception of the study. All authors read and approved the final manuscript.

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