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Protective Effects of *Aronia melanocarpa* Extract against Cyclophosphamide-Induced Delayed Toxicity on the Bladder

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ABSTRACT

Aim: *Aronia melanocarpa* is a red-purple medicinal fruit known for its therapeutic properties in the urinary system by anti-inflammatory effects with high antioxidant content. The aim of the study is to show the supportive effect of *Aronia melanocarpa* extract delayed toxicity on the bladder induced by cyclophosphamide (CYC) that an antineoplastic agent.

Material and Methods: In the study three groups were constituted control (n=7), CYC(urotoxicity group, n=7) and CYC+ARONIA(treatment group, n=7). 100 mg/kg CYC intraperitoneally were given to CYC and CYC+ARONIA groups and waited for 4 weeks to be created delayed toxicity. At the end of the 4 weeks, 200 mg/kg *Aronia melanocarpa* was administered 15 times by oral gavage every other different day to CYC+ARONIA group (1 month in total). Sacrification was performed and after serum and urine samples were taken, the bladder was released from the sphincter region with curved-tipped forceps. Bladder tissues were investigated histologically. P38 mitogen activated preotein kinase (P38 MAPK), total antioxidant (TAS) and oxidant (TOS) status were evaluated in serum and urine samples.

Results: In histology, histological damage in the bladder continued in the CYC group, while *Aronia melanocarpa* treatment caused healing in the bladder tissue in the CYC+ARONIA group. No difference was found between the groups in terms of P38 MAPK, TAS and TOS in serum and urine samples.

Conclusion: According to the experimental results, the fact that *Aronia melanocarpa* extract improves the histological damage caused by CYC in the delayed period, and the serum and urine findings were the same as the controls, brought up the therapeutic effect of *Aronia melanocarpa* in urotoxicity.

Keywords: Cyclophosphamide; *Aronia melanocarpa*; P38 MAPK; TAS-TOS.

Siklofosfamidin Gecikmiş Toksisitesine Karşı *Aronia melanocarpa* Ekstraktının Mesane Üzerine Koruyucu Etkileri

ÖZ

Amaç: *Aronia melanocarpa* yüksek antioksidan içeriğiyle antiinflamatuar etkiler göstererek üriner sistemde tedavi edici özellikleri bilinen kırmızı-mor renkli tıbbi bir meyvedir. Çalışmanın amacı antineoplastik bir ajan olan siklofosfamidin (CYC) mesanede oluşturduğu gecikmiş toksisitede *Aronia melanocarpa* ekstraktının destekleyici etkisini göstermektir.

Gereç ve Yöntemler: Çalışmada kontrol (n=7), CYC (ürotoksisite grubu, n=7) ve CYC+ARONIA (tedavi grubu, n=7) olacak şekilde üç grup oluşturuldu. CYC ve CYC+ARONIA gruplarına 100 mg/kg CYC intraperitoneal olarak 1 doz verildikten sonra gecikmiş toksisite oluşması için 4 hafta beklenildi. 4 haftanın sonunda *Aronia melanocarpa* ekstratı oral gavaj ile gün aşırı toplamda 1 ay olacak şekilde 15 kez 200 mg/kg dozunda CYC+ARONIA grubuna verildi. Sakrifikasyonda serum ve idrar örnekleri alındıktan sonra mesane dokusu sfinkter bölgesinden eğimli uçlu forsepsle serbestleştirilerek alındı. Mesane dokusu histolojik olarak değerlendirildi. Enflamatuvar belirteç olan P38 MAP Kinaz, toplam antioksidan (TAS) ve oksidan (TOS) durumu serum ve idrarda değerlendirildi.

Bulgular: Histolojide CYC grubunda mesanedeki histolojik hasar devam ederken, CYC+ARONIA grubunda *Aronia melanocarpa* tedavisi mesane dokusunda iyileşmeye sebep oldu. Serum ve idrar örneklerinde P38 MAPK, TAS ve TOS açısından gruplar arasında fark bulunmadı.

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Sonuç: Deneysel sonuçlara göre *Aronia melanocarpa* ekstraktının CYC'nin neden olduğu histolojik hasarı geç dönemde iyileştirmesi, serum ve idrar bulgularının kontrollerle aynı olması *Aronia melanocarpa*'nın ürotoksisitede terapötik etkisini gündeme getirmiştir.
Anahtar Kelimeler: Cyclophosphamide; *Aronia melanocarpa*; P38 MAPK; TAS-TOS.

INTRODUCTION

Cyclophosphamide (CYC) is an alkylating and antineoplastic agent used in solid tumors, malignancies and non-malignant diseases (1). As we know, CYC has dose-dependent side effects specific to the urinary system more common in females. These effects include dysuria, hemorrhagic cystitis, microhematuria and bladder fibrosis, necrosis, contracture (2). In the acute period, CYC causes edema, hemorrhage, leukocyte infiltration and damage, which are associated with hemorrhagic cystitis (3), as well as delayed toxic interstitial changes occur in the chronic period (4). The cause of urotoxicity is acrolein, the toxic metabolite of CYC. Acrolein induces oxidative stress by increasing lipid peroxidation, malondialdehyde (MDA) and nitric oxide (NO) levels (5) and decreasing glutathione, superoxide dismutase (SOD), catalase values (2) in the bladder (6,7) and initiates cellular damage (8). Oxidative stress (9) induced by CYC in the urothelium and detrusor smooth muscle initiates inflammation by activating the nuclear factor kappa-B (NF-KB) /P38 MAP kinase pathway (10). This triggers the release of proinflammatory cytokines and activates mast cells and leukocytes (11). Mesna is the only approved drug to detoxify CYC-induced urotoxicity (10). Therefore, there is a need to develop new therapeutic agents against the side effects of CYC, since mesna could tolerate the side effects of CYC to some extent in a dose-dependent manner (12).

It is known that blue, purple and red colored fruits with high anthocyanin content such as blueberry, raspberry and cranberry are beneficial for health (13), and have antioxidant, anti-inflammatory and antitumoral effects (14). It has been reported that anthocyanins are excreted from the urine (15), treat recurrent urinary tract infections (16), and their high antioxidant content has a chemopreventive effect (17). *Aronia melanocarpa* (black chocoberry) (18), one of the current anthocyanins, is used as food and medicine (19), and there is no data showing that it has a toxic effect (20). Studies have shown that *Aronia melanocarpa* extract reduces oxidative stress by regulating redox status in dialysis patients with anemia (21), and decreases the toxic effects of cisplatin by increasing cell viability in cisplatin-induced kidney damage in the embryonal kidney cell line (22). It has been observed that *Aronia melanocarpa* application reduces lipid peroxidation and increases lymphocyte proliferation in the immunosuppression caused by CYC in the thymus and spleen, which are immune organs other than the urinary system (23). The same researchers showed that *Aronia melanocarpa* causes a respiratory burst in neutrophils, reduces ROS products, and increases MDA levels in CYC-induced immunosuppression (24). It has been shown that *Aronia melanocarpa* extract applied to plasma collected from patients receiving CYC chemotherapy reduces oxidative/nitrative stress (25). Apparently, the high antioxidant content of *Aronia melanocarpa* fruit is good for immunosuppression caused by CYC administered as a chemotherapeutic agent and for urinary system diseases. However, any study showing the effects of *Aronia melanocarpa* extract on CYC-induced urotoxicity could not be found in the literature.

In our study, it was aimed to test a new agent with the assumption that *Aronia melanocarpa*, which has a high anthocyanin content as an alternative to mesna, will reduce inflammation and damage in the bladder against the delayed toxic effects of CYC-induced urotoxicity.

MATERIAL AND METHODS

Animals

The ethics committee of the study was obtained from Bolu Abant İzzet Baysal University (BAİBU) Experimental Animals Local Ethics Committee with the number 2022/10. In the study, Wistar albino type 2-4 months old 200-250 gr female rats were obtained from BAIBU Experimental Animals Center and maintained with ad libitum water and pallet feed under 19±2 ºC temperature and 55-60 relative humidity. In the study, 3 groups, n=7, were formed as control, CYC (cyclophosphamide-induced urotoxicity group) and CYC+ARONIA (*Aronia melanocarpa* treatment group).

Drug Administration

100 mg/kg CYC (Endoxan 500 mg intravenous infusion, Eczacıbaşı Baxter, Turkey) was dissolved in 0.2 cc saline and injected intraperitoneally (i.p) into the animal to CYC and CYC+ARONIA groups. After waiting for 4 weeks for the delayed toxicity properties to develop, *Aronia melanocarpa* extract was administered to CYC+ARONIA group 15 times every other different day at a dose of 200 mg/kg (26) in 2,5 ml saline (Pharmovit, Poland) by oral gavage (Figure 1). Control and CYC groups were given 2,5 ml saline. At the end of the study the animals were decapitated after ketamine/xylazine anesthesia (90/10 mg/kg). When the sufficient depth of anesthesia was reached under sterile conditions, the animal's abdomen was opened through a skin and subcutaneous vertical incision was made on the abdomen of the animal, which was fixed in the ventro dorsal position. After thoracotomy, 5 ml of blood was taken from the heart with the help of a syringe. Then, after 3 centimeters incision was made in the median line from the inguinal region, the syringe was entered into the bladder at 45^0 angle and sterile urine was collected. The bladder, which was released from the sphincter region with curved-tipped forceps, was dissected and removed.

ELISA

ELISA method was used in accordance with the company recommendations for the evaluation of p38 MAPK (Invitrogen, cat no: KHO0061, USA) in urine and serum samples obtained from rats/rats.

Total Antioxidant (mmol/L)-Oxidant (µmol/L) Status and Oxidative Stress Index (OSI)

The novel automated method is based on the bleaching of characteristic color of a more stable ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants. The results were expressed as mmol

Trolox equivalent/L (27). TOS and TAS levels were measured using commercially available kits (Relassay, Turkey) (28). The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). For calculation, the resulting unit of TAS was converted to μmol/L, and the OSI value was calculated according to the following Formula: OSI (arbitrary unit) = TOS (μmol H2O2 equivalent/L) / TAS (μmol Trolox equivalent/L) (29-32).

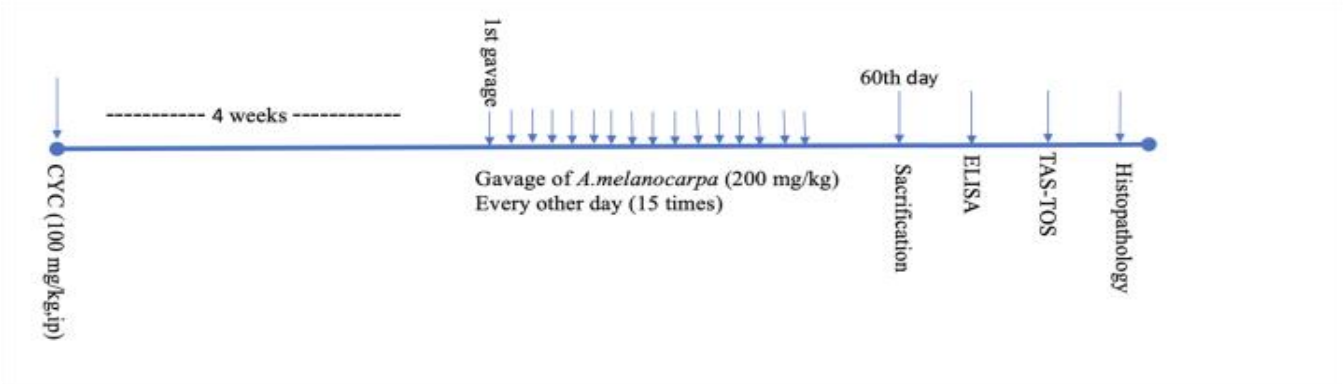

Figure1. Experimental flow chart

Histopathology

Bladder tissues taken from rats were fixed in a 10% buffered formalin solution. After fixation, tissue samples were taken into follow-up cassettes and routine tissue follow-up was performed. Serial sections of 3 μm thickness were made from the tissues embedded in paraffin blocks with a microtome, and the sections were taken on polylyzed slides. After routine deparaffinization, the sections were passed through decreasing alcohol series, and hematoxylin-eosin (H&E) staining was performed for morphological changes in the bladder (33). In the examination for bladder tissue; Inflammation, desquamated epithelium, urothelial thickness, edema, interstitial hemorrhage, vascular congestion and mast cell density were examined under the light microscope (Nikon eclipse 80i). These morphological parameters were semiquantitatively scored between 0-3. In the absence of any change, the score was 0, mild damage was evaluated as 1, moderate damage was scored 2, and severe damage was scored 3 (34).

Statistical Analysis

The data were evaluated in the IBM SPSS Statistics 25.0 (IBM Corp., Armonk, New York, USA) statistical package program. The normal distribution of the data of numerical variables was evaluated with the Shapiro Wilk test of normality. Levene's test was used to assess the homogeneity of group variances. Descriptive statistics of the data are presented as n (%) and mean±standard deviation (x±SD) if the variable is normally distributed, otherwise as median (minimum-maximum) or median (1st quartile -3rd quartile). Comparisons between groups were made with one-way analysis of variance for normally distributed variables, and Kruskal-Wallis analysis for non-normally distributed variables. Tukey HSD was used for normally distributed variables and Mann-Whitney U test with Bonferronni correction was used for non-normally distributed variables as a multiple comparison test. A p value of <0.05 was considered statistically significant.

RESULTS

Histopathology

In the light microscopic examination of H&E staining, normal histological appearance was observed in the control group (Figure 2A). When CYC group was compared with the control group, dense mast cells were found in CYC group (p=0.002). Due to thinning of the urothelium and shedding of the transitional epithelium in CYC group, bare areas consisting only of connective tissue were observed in some areas (p=0.001). In addition, interstitial hemorrhage and vascular congestion in the connective tissue area increased significantly (p<0.001) (Figure 2B). When the increase in the number of inflammatory cells between the CYC and CYC+ARONIA groups was compared, there was a statistically significant difference in inflammatory cells in CYC group (p=0.041). Bladder histology with morphology similar to the control group was observed in CYC+ARONIA group (Figure 2C). Mast cell density was significantly decreased compared to CYC group (p=0.003). While shedding of the transitional epithelium continued in CYC group (p<0.001), no significant difference was observed in the general urothelium thickness in the CYC+ARONIA group compared to the control group, which concluded that *Aronia melanocarpa* treatment was effective in urothelium healing (p=1.000) (Table 1). In addition, in CYC+ARONIA group; while interstitial hemorrhage in the connective tissue area decreased significantly (p<0.001), the decrease in vascular congestion was not statistically significant (p=0.253). With this; when examined in terms of inflammatory cell increase, a significant difference was observed between CYC and CYC+ARONIA groups (p=0.041). On the other hand, it was seen that the treatment applied was sufficient to prevent edema in the tissue (between CYC and CYC+ARONIA p=0.034) (Figure 2).

Table 1. Comparison of urothelium thickness between groups.

Histopathology	Control	CYC	CYC+ARONIA	p value
Urothelium thickness	21.4±7.44[a,b]	8.23±3.26[a,c]	18.58±7.89[b,c]	0.001*

Numeric variables are included in the table. a : There is a statistical difference between control and CYC groups. b : There is a statistical difference between control and CYC+ARONIA groups. c :There is a statistical difference between CYC and CYC+ARONIA groups.

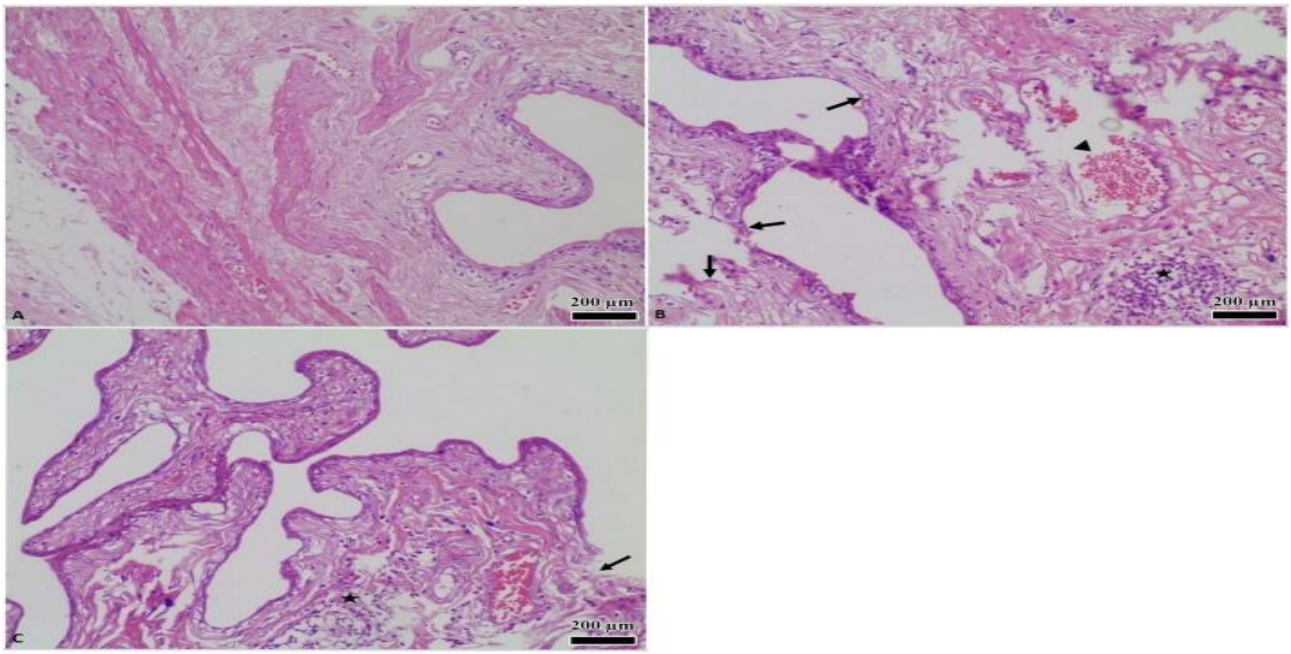

Figure 2. Control group, bladder tissue normal histological appearance (A); CYC group, desquame epithelium (arrow), vascular congestion (arrowhead), inflammation in the connective tissue area (star) (B); CYC+ARONIA group, minimal desquame epithelium (arrow), inflammation (asterisk) (C). H&E staining X200.

Total Antioxidant (mmol/L)-Oxidant (µmol/L) Status and Oxidative Stress Index (OSI)

In the measurement of oxidant stress parameters evaluated in serum samples, no significant difference was observed between the groups as a result of TAS (p=0.147),TOS (p=0.404) and OSI (p=0.206) measurements. In the measurement of oxidant stress parameters in urine samples, there was no difference between the groups in TAS (p=0.764), TOS (p=0.741) and OSI (p=0.735) values (Table 2).

Table 2. Comparison of TAS, TOS and OSI between groups.

Oxidant Stress Status		Control	CYC	CYC+ARONIA	p value
TAS (mmol/L)	**Serum**	1.57±0.25	1.40±0.15	1.55±0.11	0.147[a]
TOS (µmol/L)		19.58±6.65	27.25±13.00	19.33±6.45	0.404[a]
OSI		1.22±0.33	2.01±1.20	1.25±0.42	0.206[a]
TAS (mmol/L)	**Urine**	1.87±0.56	1.71±0.85	2.03±1.00	0.764[a]
TOS (µmol/L)		0.83±0.43	0.65±0.54	0.77±0.44	0.741[a]
OSI		0.05±0.04	0.04±0.05	0.04±0.032	0.735[a]

Numeric variables are included in the table. a: There is no difference between the groups.

ELISA

P38 MAPK protein values, which play a role in initiating the inflammatory process, were not statistically significant between the groups in both serum (p=0.320) and urine (p=0.319) samples (Table 3).

Table 3. P38 MAPK protein values between groups.

P38 MAPK (pg/mL)	Control	CYC	CYC+ARONIA	p value
Serum	0,67±0.00	0,67±0.07	0,67±0.00	0.320[a]
Urine	0.67±0.01	0.67±0.02	0.67±0.03	0.319[a]

Numeric variables are included in the table. a : There is no difference between the groups.

DISCUSSION

In this study, the effects of *Aronia melanocarpa* on histomorphology, TOS/TAS, and systemic inflammation with P38 MAPK were evaluated against late bladder damage induced by CYC. According to our results, *Aronia melanocarpa* improved against the late damage in the bladder caused by CYC; there was no difference between the treatment group and the control group in total oxidant and antioxidant status, serum and urine inflammatory P38 MAPK.

Urotoxicity can occur after both long- and short-term treatment of CYC (35). Acute hemorrhagic cystitis (36), which is caused by initiating inflammation by stimulating the P38 MAPK/NF-KB pathway by triggering oxidant stress in the first 12-hour acute period of CYC administration in the bladder, causes cellular atypical changes and late toxicity findings such as apoptosis after 30 days in the long term (37), which causes persistent urinary tract infections (38, 39). In the study, although there was histological damage to the bladder in the long-term (60th day) in the CYC group, the inflammatory marker P38 MAPK and TAS-TOS in the urine and serum were found to be statistically the same as the Control. This situation coincides with the nephrotoxic effects caused by CYC, and histopathological damage is detected in the kidney even if serum creatinine is normal in nephrotoxicity caused by CYC (40). The absence of findings in serum and urine may not indicate healing of tissue damage.

There are many pharmacological agents with high antioxidant properties to minimize the toxic effects of CYC. For this purpose, many agents with high antioxidant activity have been studied: *Aronia melanocarpa*, which is a current phytotherapeutic plant with its strong antioxidant properties, on the urinary system were examined, it was observed that *Aronia melanocarpa* improved acute renal failure with antioxidant and cytoprotective effects in acute renal ischemia-reperfusion injury (41). It has been found that *Aronia melanocarpa* prevents renotoxicity by increasing urinary excretion against oral cadmium intake (42). *Aronia melanocarpa* is known to reduce MDA levels in the kidney in a d-galactose-induced aging model (43). In a pilot study conducted in a nursing home, it was observed that *Aronia melanocarpa* juice reduced the frequency of urinary tract infections and the dose of antibiotics used for urinary tract infections in volunteers (44). Consistent with our study results, in a study, *Aronia melanocarpa* supplementation in triathletes reduced plasma and urinary oxidative stress markers (45). Although *Aronia melanocarpa* is frequently studied in medical studies with its antioxidant, anti-inflammatory, and antiapoptotic properties in healthy tissue, there is no study in the literature evaluating its effects on acute-chronic urotoxicity. According to the results of our study, although oxidative stress markers and inflammation values in serum and urine did not differ between the groups, damage findings such as edema, inflammation, mastocyte density, epithelial desquamation in the ongoing tissue in the CYC+ARONIA. For this purpose, in our study, *Aronia melanocarpa* extract was tried for the first time in the literature to improve urotoxicity, and it was aimed to develop an alternative adjuvant therapy to mesna (12).

The experimental cystitis model induced by CYC is formed differently as acute and chronic, hemorrhagic cystitis in acute model (46), interstitial cystitis model in chronic cystitis (47) are imitated. In both types of models, different doses of CYC on consecutive days (48) are applied, as well as a single dose (49, 46) or high dose (37, 50) a cystitis model is created. There is no study in the literature evaluating how the bioavailability of CYC is affected when CYC is administered together with *Aronia melanocarpa*. For this reason, we evaluated the long-term delayed toxicity findings on the basis of interstitial cystitis by administering supplemental drugs in the late period. We recommend that researchers consider drug administration time, CYC dose, and model selection when evaluating results.

Different CYC doses, lack of acute-subacute-chronic model groups, absence of apoptosis markers, complete urinalysis, and absence of kidney histology are our limitations. However, evaluation of the long-term effects of *Aronia melanocarpa* and CYC on the bladder, and our study in female rats constitute our superiority.

CONCLUSION

In conclusion, the results of this study confirm the serious damaging effect that CYC has on the bladder of the rats, including bladder edema, inflammation and hemorrhage in the late period. Furthermore, our study shows for the first time that *Aronia melanocarpa* had a protective effect against CYC-induced urotoxicity. Definitely, our findings suggest that *Aronia melanocarpa* is a potentially effective drug for prevention and treatment of delayed toxicity of CYC-induced cystitis.

Conflict of Interest

Authors declared that there is no conflict of interest.

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Authors's Contribution: Idea/Concept: Ş.K., H.Ç., A.Ç.; Design:H.Ç., Ş.K.; Data Collection and/or Processing: H.Ç., Ş.K.; Literature Review: Ş.K., H.Ç., A.Ş.; Analysis and/or Interpretation: Ş.K., H.Ç., A.Ç.; Writing the Article: A.Ç., H.Ç., Ş.K., A.Ş.; Critical Review: A.Ç.

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