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Investigation of Protective Effects of Naringin on ECG, Cardiac Enzymes, Cardiac Histopathology and 8-OHdG Expression in Cyclophosphamide-Induced Cardiotoxicity in Rats*

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Abstract: This study aimed to investigate the protective effects of Naringin in Cyclophosphamide (CYP)-induced cardiotoxicity in rats. In the study, forty male adult Sprague Dawley rats weighing approximately 200-250 g were used. Rats were divided into 5 experimental groups as control, CYP, Naringin50+CYP, Naringin100+CYP and Naringin100. Control and CYP groups were administered to intragastric (i.g.) saline for 10 days. Also, the CYP group was given a single dose of CYP (200 mg/kg, intraperitoneal (i.p.)) on the 10th day. Naringin50+CYP and Naringin100+CYP groups were administered i.g. 50 and 100 mg/kg doses of Naringin for 10 days, respectively, and was given a single dose of CYP (200mg/kg, intraperitoneal (i.p.)) on the 10th day. Naringin100 group was administered to Naringin (100mg/kg), i.g.) for 10 days. ECG was recorded under anesthesia, and intracardiac blood samples were taken. Troponin I, creatine kinase (CK) and creatine kinase-MB (CK-MB) parameters were examined in serum samples. As a result of the necropsy, preparations were prepared by routine cardiac tissue follow-up method for histopathological examination. For the detection of DNA damage in the cardiac tissue, 8-OHdG expression was investigated. CYP has been shown to have cardiotoxic effects on ECG values, cardiac enzymes, and cardiac histopathology of rats. In this study, it was determined that Naringin has a protective effect in CYP-induced cardiotoxicity in rats.

Keywords: Cardiac enzymes, Cyclophosphamide, ECG, Naringin, Rat.

Sıçanlarda Siklofosfamid ile İndüklenen Kardiyotoksisitede EKG, Kardiyak Enzimler, Kardiyak Histopatoloji ve 8-OHdG Ekspresyonu Üzerine Naringin'in Protektif Etkilerinin Araştırılması

Öz: Bu çalışmada, sıçanlarda Siklofosfamid (CYP) ile indüklenen kardiyotoksisitede Naringin'in koruyucu etkilerinin araştırılması amaçlandı. Çalışmada, ortalama 200-250 gr ağırlığında kırk adet erkek yetişkin Sprague Dawley sıçan kullanıldı. Sıçanlar kontrol, CYP, Naringin 50+CYP, Naringin100+CYP ve Naringin100 olmak üzere 5 deney grubuna ayrıldı. Kontrol ve CYP gruplarına, 10 gün intragastrik (i.g.) serum fizyolojik uygulandı. Ayrıca, CYP 10. gün tek doz CYP (200 mg/kg, intraperitoneal (i.p.)) verildi. Naringin 50+CYP ve Naringin100+CYP gruplarına, 10 gün boyunca sırasıyla 50 ve 100 mg/kg dozunda i.g. Naringin verildi ve 10. gün tek doz CYP (200 mg/kg, i.p.) verildi. Naringin100 grubuna ise, 10 gün 100 mg/kg dozunda i.g. Naringin verildi. Anestezi altında EKG kaydı yapıldı ve intrakardiyak kan örnekleri alındı. Serum örneklerinde Troponin I, kreatin kinaz (CK) ve kreatin kinaz-MB (CK-MB) parametreleri incelendi. Otopsi sonucunda histopatolojik inceleme için rutin kalp dokusu takip yöntemi ile preparatlar hazırlandı. Kalp dokusunda DNA hasarının tespiti için 8-OHdG ekspresyonu araştırıldı. CYP'nin sıçanların EKG değerleri, kardiyak enzimleri ve kardiyak histopatolojisi üzerinde kardiyotoksik etkileri olduğu gösterilmiştir. Bu çalışmada, sıçanlarda CYP ile indüklenen kardiyotoksisitede Naringin'in koruyucu etkilere sahip olduğu belirlendi.

Anahtar Kelimeler: EKG, Kardiyak enzimler, Naringin, Sıçan, Siklofosfamid.

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INTRODUCTION

Cyclophosphamide (CYP) is an antineoplastic chemotherapeutic agent and is a commonly used anticancer and immunosuppressant drug (1). CYP is also commonly used to treat acute and chronic leukemia, breast cancer, multitype myeloma, lymphoma, rheumatoid arthritis, and bone marrow transplantation (2-4). Antineoplastic chemotherapeutic agents generally have cytotoxic effects on one or more target tissues (5). The main side effects of CYP are renal toxicity (6), hematopoietic depression and hemorrhagic cystitis (7). Two active metabolites of CYP are phosphoramide and acrolein (6). The toxic effect of CYP is related to acrolein which is an active metabolite of it. Acrolein interferes with the tissue antioxidant defense system leading to be high free radical formation (4,6). Recently, the number of studies using herbal compounds has been increasing to minimize the cytotoxic effects of antineoplastic drugs (8,9). One of the compounds used for this purpose is Naringin. Naringin is a vegetable flavonoid and has had antiulcer, aortic distillation, a superoxide scavenger, and antioxidant activities (10,11). Numerous studies have been conducted to inspect the effects of cancer and CYP on the treatment process. However, to eliminate the side effects caused by CYP, a sufficient number of direct literature studies have not been found to investigate the protective effects of Naringin on the histopathology of the heart, DNA damage in the heart tissue, ECG, and heart enzymes. This study; it is aimed to investigate the effects of Naringin on ECG, heart enzymes, cardiac histopathology, and DNA damage (8-OHdG) in CYP-induced cardiotoxicity in rats and contribute to the literature.

MATERIALS AND METHODS

Animals

The animal material of this study was obtained from Atatürk University Medical Experimental Research and Application Center. The animals were cared for under appropriate conditions during the study, and all applications on animals were performed in this center. This experimental study was approved by the Local Ethics Committee for Animal Experiments of Atatürk University (Protocol No: 2016/125).

Experimental Procedure

In this study, 40 male Spraque Dawley rats weighing approximately 200-250 g were used. Eight rats in each group were used, and 5 groups were formed. The control group received saline (1 ml) intragastric (i.g.) for 10 days. CYP group was given i.g. serum physiologic for 10 days and then was given intraperitoneal (i.p.) CYP (200 mg/kg) in a single dose on 10^{th} day. Naringin50+CYP and Naringin100+CYP groups were given in serum physiologic solubilized Naringin with 50 and 100 mg/kg for 10 days, respectively, and then CYP (200 mg/kg, i.p.) in a single dose was administered on the 10^{th} day after naringin injection. Naringin100+CYP group was given 100 mg/kg (i.g.) solubilized in serum physiologic naringin for 10 days. Forty-eight hours after the CYP administration (2,4,6), ECGs of all rats were recorded as bipolar limb leads (I, II and III) and unipolar limb leads (aVR, aVL, and aVF) (12-14). The heart rate, amplitude, and duration of waves in leads II, the mean electrical axis of heart leads I, and III were determined (15-17). After ECG was recorded, intracardiac blood samples were taken under anesthesia with rats thiopental sodium (20 mg / kg) and rats sacrificed by cervical dislocation method. Troponin I's levels were tested by a commercial ELISA kit and analyzed according to the manufacturer's protocol. CK and CK-MB's levels were determined a Modular pp auto analyzer.

After necropsy, the preparations of cardiac tissues were examined under a light microscope. The sections were evaluated as none (-), mild (+), moderate (++) and severe (+++) according to the lesions, and the pictures were taken. For the

detection of DNA damage, 8-OHdG antibody kit and AbcamHRP/DAB Detection IHC kit were used according to the kits procedure. 3-3 'Diaminobenzidine (DAB) was used as the chromogen. Sections were evaluated as absent (-), mild (+), moderate (++), and severe (+++) according to their immune positivity.

Statistical Analysis

SPSS 20.0 package program was used for statistical analysis. ANOVA and Duncan's test were used to evaluate the statistical differences obtained of data at the end of the studies. In the histopathological examination, the non-parametric Kruskal-Wallis test and Mann-Whitney U test were used to compare the differences between the groups.

RESULTS

The ECG values of the rats in the control and experimental groups in Table 1 and the ECG samples in Figures 1, 2, 3, and 4, histopathological and immunohistochemical appearance of the heart tissue in Figures 5 and 6 were presented, and the scoring of histopathological and immunohistochemical findings in the heart tissue in Table 3 was given. As shown in Table 1, rat's heart rate in the control and CYP groups were 375 ± 30 and 510 ± 17, respectively. CYP caused sinus tachycardia. Arrhythmias, sinus tachycardia, myocardial infarction and ST elevations characterized by secondary cardiomyopathy were observed in the ECG's of CYP administrated rats (Figure 2-4). Elevated levels of cardiac enzymes (Troponin I, CK, and CK-MB) in the CYP group also support property these findings (Table 2). Besides, in some rats, second-degree heart blocks were detected (Figure 3). Naringin 50 and 100 mg/kg doses seem to reduce sinüs tachycardia caused by CYP but not wholly prevent (Table 1). The values in Naringin 100 group are similar to the control group.

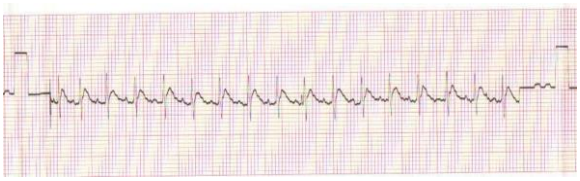

Figure 1. Electrocardiography in the control group rats (1 mV = 10 mm, 50 mm/sec).
Şekil 1. Kontrol grubundaki ratlarda elektrokardiyografi (1 mV = 10 mm, 50 mm/sec).

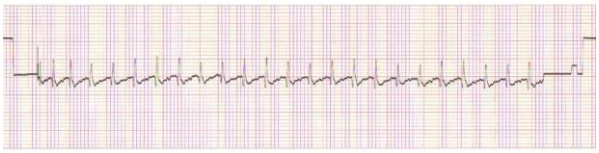

Figure 2. Sinus tachycardia in the CYP group rats (1 mV = 10 mm, 50 mm/sec).
Şekil 2. CYP grubundaki ratlarda sinüs taşikardi (1 mV = 10 mm, 50 mm/sec).

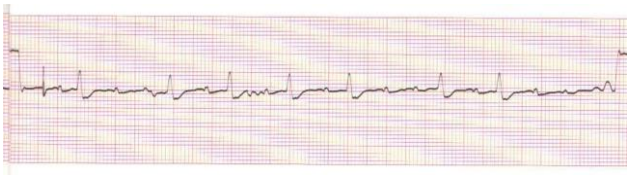

Figure 3. Second-degree heart block in the CYP group rats (1 mV=10 mm, 50 mm/sec).
Şekil 3. CYP grubundaki ratlarda ikinci derece kalp bloğu (1 mV = 10 mm, 50 mm/sec).

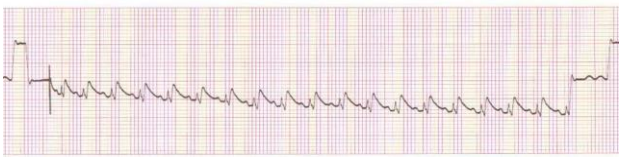

Figure 4. ST elevation in the CYP group rats (1 mV = 10 mm, 50 mm/sec).
Şekil 4. CYP grubundaki ratlarda ST yükselmesi (1 mV = 10 mm, 50 mm/sec).

The duration and amplitude of the P wave, T wave, PQ interval, QT interval, and QRS complex in ECG traces were shorter in the CYP group than the Control and Naringin 100 groups. This reduction was found to be significant at $P<0.05$ level. The hearts's mean electrical axis varied between 52±100 and 55±90 in rats in the control and experimental groups, and there was no statistically significant difference among the groups.

Table 1. The amplitude and duration of waves in leads II of rats in the experimental groups (Mean ± SEM, n=8).
Tablo 1. Deney gruplarındaki sıçanların II. Derivasyonundaki dalgaların genliği ve süresi (Ortalama±Standart Sapma, n=8).

ECG's Parameters	Groups Control	CYP	Naringin50+CYP	Naringin100+CYP	Naringin 100
P (s)	0.024± 0.00 [a]	0.019± 0.00 [b]	0.019± 0.00 [b]	0.020± 0.00 [b]	0.024±0.00 [a]
P (mV)	0.1± 0.00	0.1± 0.00	0.1± 0.00	0.1± 0.00	0.1± 0.00
P-Q (s)	0.04± 0.00 [a]	0.03± 0.00 [b]	0.03± 0.00 [b]	0.03± 0.00 [b]	0.04± 0.00 [a]
QRS (s)	0.042± 0.00 [a]	0.027± 0.00 [b]	0.029± 0.00 [b]	0.029± 0.00 [b]	0.042± 0.00 [a]
QRS (mV)	0.5± 0.00 [a]	0.4± 0.00 [b]	0.4± 0.00 [b]	0.5± 0.00 [a]	0.5± 0.00 [a]
QT (s)	0.053± 0.00 [a]	0.042± 0.00 [b]	0.042± 0.00 [b]	0.043± 0.00 [b]	0.053± 0.00 [a]
T (s)	0.044± 0.00 [a]	0.033± 0.00 [b]	0.034± 0.00 [b]	0.035± 0.00 [b]	0.044± 0.00 [a]
T (mV)	0.1± 0.00	0.1± 0.00	0.1± 0.00	0.1± 0.00	0.1± 0.00
Hearth rate (minute)	375±30[a]	510±17[b]	494±28[ab]	475±27[ab]	385±26[a]
Electrical axis (degree)	54±11 [a]	53±9 [a]	55±9 [a]	52±10 [a]	54±8 [a]

[a b]; Means in the same row with different superscripts differ significantly (P<0.05), CYP(cyclophosphamide), P(P wave), P-Q (P-Q wave), QRS(QRS wave), QT(QT wave), T(T wave).

As can be seen in Table 2, Troponin I, CK, and CK-MB levels were found to be statistically significant between the control group and the CYP group at P<0.001. These parameters were lower in Naringin 100+CYP group than Naringin50+CYP group (P<0.05). There was no statistically significant difference between the control group values and Naringin 100 group values, and the values were very close to each other.

Table 2. Serum Troponin I, CK and CK-MB levels in experimental groups (Mean ± SEM, n=8).
Tablo 2. Deney gruplarında Serum Troponin I, CK ve CK-MB seviyeleri (Ortalama±Standart Sapma, n=8).

Groups	Troponin I (ng/ml)	CK (U/L)	CK-MB (U/L)
Control	0.5±0.1[a]	156±16[a]	284±54 [a]
CYP	11±2[b]	368±64[b]	1574± 166[b]
Naringin50+CYP	10.3±2.3[b]	358±42[b]	1362±107[b]
Naringin100+CYP	7±2.5[c]	207±15[c]	1040± 147[c]
Naringin100	0.5±0.1[a]	153± 19[a]	278±47[a]

Means in the same column with different superscripts differ significantly, ab, ac: P<0.001, bc: P<0.05, CYP(cyclophosphamide).

Epicardium, myocardium and endocardium were found to have normal histological structure (Figure 5 A-E) and determined negative 8-OHdG expression (Figure 6A and 6E) in the Control and Naringin 100 groups. The CYP group, showed impaired hyaline degeneration, Zenker's necrosis, interstitial hyperemia (Figure 5B), and detected severe 8-OHdG expression in the muscle fibers (Figure 6B). In the Naringin 50+CYP group, moderate hyaline degeneration in the muscle fibers and hyperemia in the interstitial vessels were detected (Figure 5C), and moderate 8-OHdG expression was observed (Figure 6C). In the Naringin 100+CYP group, mild hyaline degeneration in the muscle fibers and hyperemia in the interstitial vessels were detected (Figure 5D), and mild 8-OHdG expression was found (Figure 6D). Histopathological and immunohistochemical findings were summarized in Table 3.

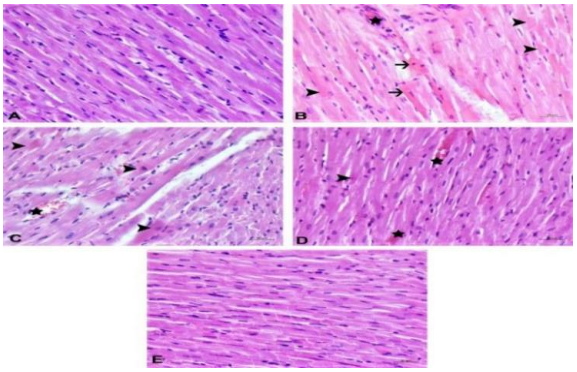

Figure 5. Heart muscle, In the Control and Naringin 100 groups, normal histological appearance (AE), In the CYP group, disrupted saturation, hyaline degeneration (arrows), Zenker's necrosis (arrowheads) in the muscle fibers, hyperemia in the interstitial vessels (stars) (B), In the Naringin50+CYP groups, moderate hyaline degeneration (arrows), hyperemia in the interstitial vessels (stars) (C), In the Naringin100+CYP groups, mild hyaline degeneration (arrows), hyperemia in the interstitial vessels (stars) (D), H&E, Bar: 20µm.
Şekil 5. Kalp kası, Kontrol ve Naringin 100 gruplarında normal histolojik görünüm (AE), CYP grubunda bozulmuş satürasyon, hiyalin dejenerasyonu (oklar), kas liflerinde Zenker nekrozu (ok başları), interstisyel damarlarda (yıldız) hiperemi (B), Naringin 50 + CYP

gruplarında orta derecede hiyalin dejenerasyonu (oklar), interstisyel damarlarda hiperemi (yıldız) (C), Naringin 100 + CYP gruplarında, hafif hiyalin dejenerasyonu (oklar), interstisyel damarlarda hiperemi (yıldız) (D), H&E, Bar: 20µm.

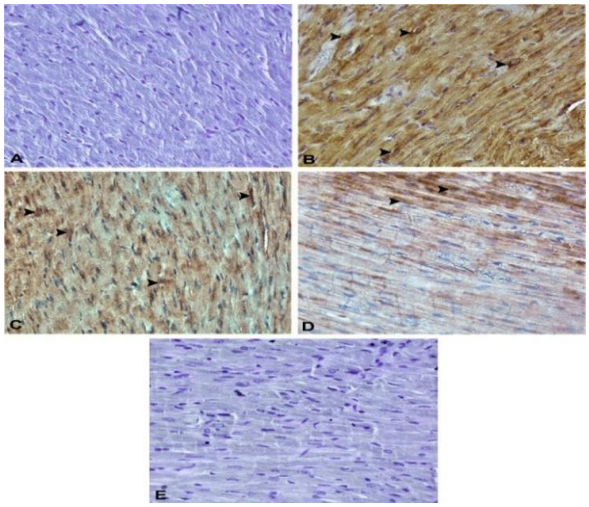

Figure 6. Heart muscle, In the Control and Naringin100 groups; negative 8-OHdG expression (A, E), In the CYP group; severe 8-OHdG expression (arrowheads) in muscle fibers (B), In the Naringin50+CYP groups; moderate 8-OHdG expression (arrowheads) in muscle fibers (C), In the Naringin100+CYP groups; mild 8-OHdG expression (arrowheads) in muscle fibers (D), IHC-P, Bar: 20µm.
Şekil 6. Kalp kası, Kontrol ve Naringin 100 grubunda; negatif 8-OHdG ifadesi (A, E), CYP grubunda; kas liflerinde şiddetli 8-OHdG ekspresyonu (ok başları) (B), Naringin50+CYP gruplarında; Kas liflerinde orta düzeyde 8-OHdG ifadesi (ok uçları) (C), Naringin100+CYP gruplarında; kas liflerinde hafif 8-OHdG ifadesi (ok başları) (D), IHC-P, Bar: 20µm.

Table 3. Scoring of histopathological and immunohistochemical findings in cardiac muscle.
Tablo 3. Kalp kasında histopatolojik ve immunhistokimyasal bulguların skorlanması.

Groups	Hyaline Degeneration	Zenker's Necrosis	Hyperemia in Veins	8-OHdG Expression
Control	-	-	-	-
CYP	+++	+++	+++	+++
Naringin50+CYP	++	+	++	++
Naringin100+CYP	+	-	+	+
Naringin100	-	-	-	-

none (–), mild (+), moderate (++), and severe (+++), CYP(cyclophosphamide)

DISCUSSION and CONCLUSION

CYP is an anticancer agent that is commonly used in chemotherapy (1). CYP is also a good mutagen and clastogen (4,5). As a result of the studies, patients treated with CYP have side effects such as anemia, neutropenia, vomiting, nausea, skin lesions, mouth sores, cystitis, and absence of menstrual cycle. CYP also causes gonadal toxicity and oxidative stress in the liver (2,6,18). High doses of CYP cause acute cardiotoxicity, such as cardiac decompensation with fatal cardiomyopathy (1,6,7,19). Some antioxidant compounds are used to reduce oxidative stress and the possible side effects of anticancer drugs (2,8-10,20). Naringin is one of the potent antioxidant compounds that are abundant in citrus fruits. There is not enough literature about the effects of Naringin on CYP-induced cardiotoxicity in rats.

The values for the duration and amplitude of the P wave and the PQ interval in the Control and Naringin 100 groups are consistent with the literature (12-15). P wave duration reported should be approximately 1/3 or 1/2 of the duration of the PR interval, which is consistent with the findings in this study (21,22). The QRS value of the rats in the CYP group was lower than in the Control and Naringin 100 groups. The reason for the fall was due to the high heart rate and sinus tachycardia in the CYP group. In the ECG's of the CYP applied rats have observed arrhythmias, sinus tachycardia, myocardial infarction, and ST elevations characterized by secondary cardiomyopathy. In the CYP group was detected impaired saturation in muscle fibers, hyaline degeneration, Zenker's necrosis, hyperemia on interstitial veins, severe 8-OHdG expression and increase cardiac enzymes (Troponin I, CK and CK-MB)

levels are important, this datas are supporting the literature (4,6,23,24).

Increased levels of Troponin I and CK-MB and detection of 8-OHdG expression are even more important in cardiac muscle damage. While CK increases in heart and whole skeletal muscle damage, Troponin I and CK-MB increase especially in heart muscle damage, and this increase is significant in the differential diagnosis. If the ratio of CK-MB to total CK is greater than 2.5-3 %, the risk of heart damage is high, and this is an indicator of heart muscle damage. CK and CK-MB values in the control and Naringin100 groups in our study are similar to those determined by Comba et al. (25). Troponin I, CK, CK-MB, and 8-OHdG expression were found to be significant between the control group and CYP group. These parameters were lower in the Naringin100+CYP group than the Naringin50+CYP group, and this decrease was significant between the two groups. There was no statistically significant difference between the Control group values and Naringin 100 group values, and these values were very close to each other both in histopathological, biochemical, and immunohistochemical contexts. In the CYP group, raised cardiac enzymes, increased 8-OHdG expression, impaired saturation in muscle fibers, hyaline degeneration, Zenker's necrosis and ST elevations were indicated to be secondary cardiomyopathy, and this is consistent with the literature (14,26-28). In addition, some rats were detected to the second-degree heart blocks. In a study, it was reported that CYP causes left bundle branch block in dogs (29). In another study, it was described that CYP causes dose-dependent cardiotoxicities, and this situation can reach up to fatal cardiomyopathy (26). Symptoms of CYP-induced cardiotoxicity often occur within 1 or 3 weeks. Previous treatment with anthracycline group medication and radiation, being over 50 years of age, and left ventricular dysfunction are among the risk factors for CYP-induced cardiotoxicity (29). Naringin was used in our study to eliminate the cardiotoxic side effects of CYP. Naringin 50 and 100 mg/kg doses were reduced to CYP's causes sinüs tachycardia, heart blocks, and ST elevation but not completely prevented. The PQ and QT interval represents the auricular and ventricular activation time, respectively (30-32). PQ and QT values were lower in the CYP group than in the Control and Naringin 100 groups, and a significant difference was found among the groups. Naringin was prevented from shortening in PQ and QT intervals. Long QT syndrome associated with syncope and sudden cardiac death is a risk factor for fatal ventricular arrhythmias. Shortening of QT interval in tachycardia, prolongation of QT interval in cases of electrolyte imbalances (hypopotassemia, hypomagnesemia), and bradycardia may cause type ventricular arrhythmias (29).

In this study, the number of minute heartbeats was 375 in the control group, 510 in the CYP group, 494 in the Naringin50+CYP group, 475 in the Naringin100+CYP group and 385 in the Naringin100 group. The heart rate in the CYP group was statistically significant at compared to the control group and Naringin100 group. These values obtained from our research are consistent with 410 values stated by Bulduk and Kılıçalp (15).

The T wave duration in the CYP group was significantly lower than the value of the Control and Naringin100 group, at a statistically significant. This decrease is due to the side effects of CYP. The duration of the T wave in the control and Naringin 100 groups was consistent with the values reported for rats (14,17). The electrical axis of the heart is + 54°, + 53°, + 55°, 52° and + 54° in the groups, respectively. This can be interpreted to mean that the heart is set forward and slightly to the left in rats. The average electrical axis of the heart, Yilmaz (13) reported an average of 28^0, while Cieslar et al. (32) stated 33^0 degrees on average. The results obtained from rats in both control and experimental groups support the above researchers.

The results of this research showed that, to some extent, Naringin has had protective effects on the cardiovascular system in CYP-induced

cardiotoxicity in rats. In addition, we hope to contribute to literature for future scientific studies.

Conflict of interest

The authors declare that they have no conflict of interest.

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