EXPERIMENTAL STUDY

Comparison of the protective effects of vanillic and rosmarinic acid on cardiac tissue: Lower limb ischemia-reperfusion model in rats

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ABSTRACT

BACKGROUND: Ischemia/reperfusion injury is one of the most challenging postoperative situations in vascular surgery, both in elective procedures with prolonged clamping time and in delayed emergency cases with vascular occlusion. The inflammatory response that develops during ischemia and the oxygen-free radicals that proliferate during reperfusion have detrimental effects on the brain, heart, and kidneys. In this study, we aimed to compare the effects of vanillic and rosmarinic acid in preventing ischemia/reperfusion injury in a lower limb ischemia-reperfusion model in rats.

METHODS: Thirty-two female Sprague-Dawley rats weighing 185-240 g were randomly divided into four groups of eight animals each. Group 1 was designated as the control, Group 2 as ischemia/reperfusion (I/R), Group 3 as ischemia/reperfusion + vanillic acid (I/R + VA), and Group 4 as ischemia/reperfusion + rosmarinic acid (I/R + RA). In all groups except the control, the infrarenal abdominal aorta was clamped, and 60 minutes of ischemia followed by 120 minutes of reperfusion was performed. Vanillic acid was administered intra-abdominally 15 minutes before the start of reperfusion in Group 3, and rosmarinic acid in Group 4. At the end of the reperfusion phase, blood samples and hearts were collected, and the rats were euthanized. Histopathologically, myofibrillar edema, myocytolysis, focal hemorrhages, and infiltration of polymorphonuclear leukocytes (PMNL) in cardiac tissue were examined. Total antioxidant capacity (TAC), total oxidative status (TOS), oxidative stress index (OSI), 8-OH-deoxyguanosine, lactonase, and arylesterase activity were measured in blood samples.

RESULTS: Myofibrillar edema was most pronounced in the I/R group and less pronounced in the I/R + VA and I/R + RA groups ($p=0.005$ and $p=0.066$, respectively). There was no difference between the ischemia/reperfusion groups regarding myocytolysis, focal hemorrhage, and PMNL infiltration ($p>0.99$). Among all groups, TOS and OSI were lowest in the control group, while TAC was highest. TAC was similar in the I/R + VA and I/R + RA groups but was significantly higher in these two groups than in the I/R group. The lactonase activity in the I/R + VA group was similar to that in the control group but was significantly higher compared to the I/R and I/R + RA groups.

CONCLUSION: Our study shows that vanillic and rosmarinic acids reduce myofibrillar edema in the heart after lower limb ischemia and increase TAC. However, vanillic acid increases the activity of lactonase, an enzyme known for its antioxidant effect, more than rosmarinic acid.

Keywords: Cardiac; ischemia; lactonase; reperfusion; rosmarinic; vanillic.

Cite this article as: Huseyin S, Reyhancan A, Halici U, Guclu O, Tuysuz S, Oztorun B, et al. Comparison of the protective effects of vanillic and rosmarinic acid on cardiac tissue: Lower limb ischemia-reperfusion model in rats. Ulus Travma Acil Cerrahi Derg 2024;30:619-625.

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Ulus Travma Acil Cerrahi Derg 2024;30(9):619-625 DOI: 10.14744/tjtes.2024.12359

Submitted: 07.05.2024 Revised: 24.07.2024 Accepted: 25.07.2024 Published: 02.09.2024

INTRODUCTION

Endovascular interventions for diseases of the abdominal aorta and iliac arteries are now widely and successfully used in many clinics. However, for patients with low risk and high life expectancy, traditional surgery is still considered the gold standard.[1] Clamping the aorta during surgery leads to reduced blood flow to the lower extremities and bowel, resulting in ischemic conditions. During ischemia, certain chemical mediators occur due to oxidative stress. After the clamp is released, reoxygenation occurs, leading to the formation of oxygen-free radicals (OFRs) that can enter the bloodstream and cause distant organ damage. Both the gastrointestinal tract and the lower extremities contribute to a generalized inflammatory response.[2] After abdominal aortic surgery, myocardial damage and dysfunction may occur, which is an important cause of mortality and morbidity.[3] In addition, ischemia/reperfusion injury (IRI) can lead to multiple organ failure.[4]

The use of agents that both increase antioxidant capacity and reduce the production of reactive oxygen species (ROS) in tissue undergoing ischemia has received considerable attention in animal studies. For this purpose, drugs, herbs, and fruit extracts were administered to rats, and various tissues (kidney, lung, heart, etc.) were analyzed.[5-11]

Vanillic acid (VA) is found in edible and some medicinal plants (such as Angelica sinensis), beverages like tea and coffee, and fermented alcoholic beverages including wine and beer. It is also used as a flavoring and preservative in packaged foods.[5] Rosmarinic acid (RA) occurs naturally in common plants in nature, such as mint, sage, and basil. It has a molecular structure with two phenolic rings and can be extracted from lemon balm plants.[6,7]

In the present study, we aimed to investigate the effects of VA and RA on reperfusion-induced cardiac tissue injury in an experimental model of lower limb IRI.

MATERIALS AND METHODS

In our study, 32 female Sprague-Dawley rats, aged 3.5-4 months and weighing 185-240 g, were used. The rats were randomly divided into four groups of eight rats each. The abdominal aorta was clamped at the infrarenal level to produce ischemia of the lower extremities. After 60 minutes of clamping, the clamp was removed and a 120-minute reperfusion period was initiated. No additional agent was administered in the control and ischemia-reperfusion groups, while VA and RA were administered in the remaining groups. Cardiac tissue samples were examined with a light microscope, and histopathologic assessment was performed according to Suzuki's criteria. Outcomes were compared between the groups.

The study was approved by the local ethics committee (No. 2022/08) and conducted in the laboratory animal unit. The experimental procedures were performed in accordance with the Animal Welfare Act and relevant guidelines, and the animals were used in accordance with the Guide for the Care and Use of Laboratory Animals.

Sample Preparation

All rats were administered intramuscular anesthesia consisting of 40 mg/kg ketamine hydrochloride (HCl) and 5 mg/kg xylazine hydrochloride into the muscles of the left forelimb after 8 hours of fasting. Care was taken to ensure that the rats breathed continuously throughout the procedure. The rats were positioned supine on the table and irradiated with a heat lamp. A median laparotomy was performed under aseptic conditions, and the intestines were diverted to the right side. Subsequently, all rats were administered 100 U/kg of heparin intravenously via the infrarenal abdominal aorta. A vascular clamp was then applied to the infrarenal aorta, and 5 ml of warm saline was injected into the abdominal cavity. The two sides of the abdominal incision were approximated to prevent fluid and heat loss. After one hour, the vascular clamp was removed, and a two-hour reperfusion period was initiated. In rats to be administered drugs, VA and RA were administered intraperitoneally 15 minutes before the start of the reperfusion period. After the experiment, the rats were euthanized, and the removed tissues (hearts) were stored in a 10% formaldehyde solution.

Experimental Groups

Group 1: Control (n=8)

All surgical procedures except ischemia/reperfusion were performed on this group of rats as in the other groups, and blood samples and hearts were collected after surgery.

Group 2: Ischemia/Reperfusion (I/R) (n=8)

This group of rats was subjected to 60 minutes of ischemia and 120 minutes of reperfusion without the administration of VA and/or RA. After reperfusion, blood samples and hearts were collected.

Group 3: Ischemia/Reperfusion + Vanillic acid (I/R + VA) (n=8)

Rats received 12 mg/kg VA intraperitoneally 15 minutes before clamp removal and the start of reperfusion, and blood samples and hearts were collected after reperfusion.

Group 4: Ischemia/Reperfusion + Rosmarinic acid (I/R + RA) (n=8)

Rats received 50 mg/kg of RA intraperitoneally 15 minutes before clamp removal and the start of reperfusion. Blood samples and hearts were collected after reperfusion.

Histopathological Examination and Scoring

The heart tissue samples were individually fixed in 10% formalin. Kerosene blocks were then prepared from these samples, and 5 µm thick sections were cut and stained with hematoxylin and eosin (H&E). Histopathologic assessment was performed under a light microscope, focusing on parameters such as myofibrillar edema, myocytolysis, focal hemorrhage, and polymorphonuclear leukocyte (PMNL) infiltration, each

of which was scored from 0 to 4. The changes were categorized as follows:
- 0: No change detected.
- 1: Mild damage
- 2: Moderate damage
- 3: Severe damage.

Biochemical Examination

The blood samples taken from the rats were placed in yellow tubes that contained no coagulation activator. The coagulation process of the samples was completed. They were then separated into serum by centrifugation at 3000 g for 10 minutes. The collected samples were aliquoted and stored at −80°C until the next working day. Total oxidative status (TOS) and total antioxidant capacity (TAC) were determined spectrophotometrically in the samples brought to room temperature on the working day. The oxidative stress index (OSI) was calculated by the ratio of TOS and TAC.

Statistical Analysis

Based on the study by Bakar et al.,[11] an effect size of 0.85 was determined, and it was assumed that including a total of 24 observations in the study would provide sufficient statistical power of 80% at a significance level of 5%. However, taking into account an expected loss of 30%, it was decided to include 32 observations in the study.

The normality of the histopathological and biochemical measurements was assessed using the Shapiro-Wilk test. Subsequently, the variables in the study are presented primarily as median values (minimum-maximum) and supplemented by means and standard deviations (SD) based on the results of the normality test.

The Kruskal-Wallis test was used to compare variables between all groups. The Mann-Whitney U test was used for pairwise comparisons of groups. SPSS software (IBM Corp., Released 2015. IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY: IBM Corp.) was used for statistical analysis. The significance level was set at $p<0.05$.

RESULTS

In the I/R group, severe congestion, cytoplasmic vacuolization, and parenchymal necrosis were found in over 60% of cases (score 4). On the other hand, moderate congestion, cytoplasmic vacuolization, and parenchymal necrosis were observed in less than 60% of cases (score 3) in both the I/R + VA and I/R + RA groups; mild congestion, cytoplasmic vacuolization, and parenchymal necrosis were observed in less than 30% (score 2). Myofibrillar edema was mainly observed in the I/R group; there was no significant difference between the I/R + VA and I/R + RA groups. The light microscopic images of the cardiac tissue are shown in Figure 1. Myocytolysis was least pronounced in the control group, and no significant differences were observed between the other three groups exposed to ischemia. There was no significant difference between the groups in terms of focal hemorrhage and polymorphonuclear leukocyte infiltration ($p>0.05$). Comprehensive results and comparisons of the histopathologic examination can be found in Table 1.

The control group had the lowest TOS and OSI and the highest TAC ($p<0.05$). Conversely, the I/R + RA group had the highest TOS, while the I/R + VA group had the lowest OSI of all groups except the control group. It is noteworthy that the TOS and OSI values were significantly lower in the I/R + VA group than in the I/R + RA group. However, no significant difference was found between these two groups in terms of TAC ($p>0.05$).

When comparing arylesterase activity, no significant differ-

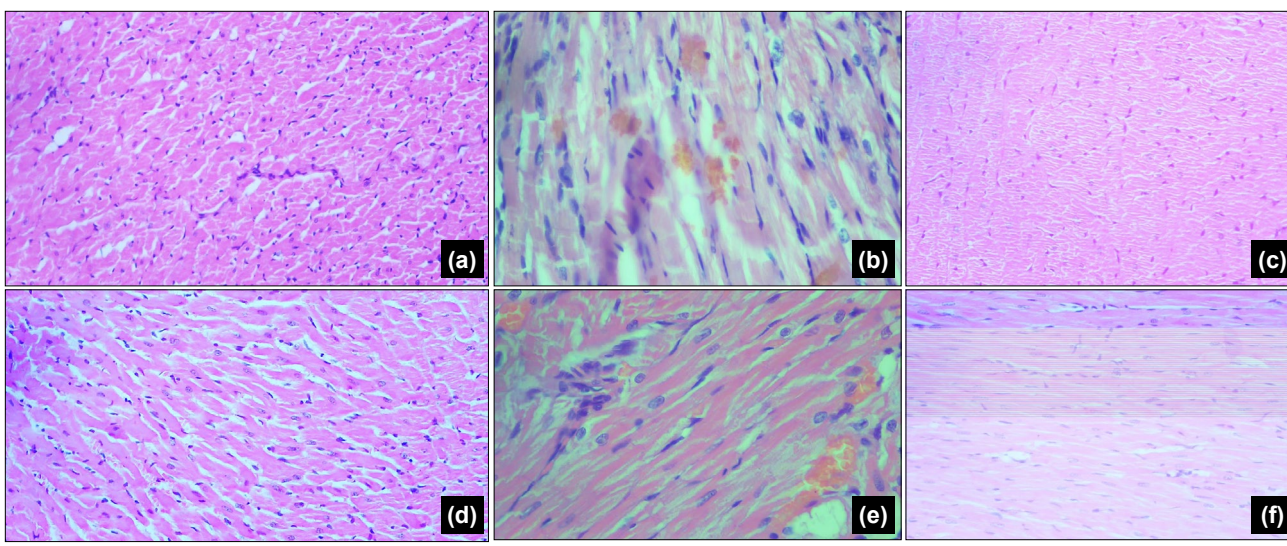

Figure 1. (a). Control group: normal myofibrils. **(b)** I/R group: Severe myofibrillar edema and hemorrhage. **(c)** I/R + VA group: Mild myofibrillar edema. **(d)** I/R + VA group: Moderate myofibrillar edema. **(e)** I/R + RA group: Moderate edema and hemorrhage. **(f)** I/R + RA group: Moderate myofibrillar edema.

Table 1. Histopathological examination results

	Control (n=8)	I/R (n=8)	I/R + VA (n=8)	I/R + RA (n=8)	p*
Myofibrillar Edema	0.25±0.46	2.38±0.52	1.38±0.52	1.63±0.92	<0.001
	0 (0-1)	2 (2-3)	1 (1-2)	1 (1-3)	
Myocytolysis	0.38±0.52	1±0	1±0	1±0	0.001
	0 (0-1)	1 (1-1)	1 (1-1)	1 (1-1)	
Focal Hemorrhage	1.25±0.46	1.5±0.76	1.88±0.84	1.25±0.46	0.263
	1 (1-2)	1 (1-3)	2 (1-3)	1 (1-2)	
PMNL Infiltration	0.88±0.64	1±0	1±0	1±0	0.785
	1 (0-2)	1 (1-1)	1 (1-1)	1 (1-1)	

PMNL: Polymorphonuclear Leukocytes. *Kruskal-Wallis test.

Table 2. Biochemical test results

	Control (n=8)	I/R (n=8)	I/R + VA (n=8)	I/R + RA (n=8)	p*
TOS (µmol H2O2 Eq/L)	10.17±3.40	18.46±3.75	14.54±4.98	22.64±3.88	<0.001
TAC (mmol Trolox Eq/L)	1.56±0.19	1.03±0.20	1.29±0.23	1.29±0.17	0.001
OSI (TOS/TAS)	0.69±0.36	1.86±0.53	1.18±0.52	1.78±0.33	0.001
Arylesterase Activity (IU/L)	51.07±4.82	47.12±5.04	43.85±7.41	46.83±4.72	0.194
Lactonase Activity (IU/L)	7.10±0.90	6.29±1.44	8.24±0.98	6.74±1.52	0.020
8-OHdG (ng/mL)	6.23±1.04	7.12±1.20	6.39±1.47	7.08±1.25	0.329

8-OhdG: 8-Hydroxy-2'-Deoxyguanosine; OSI: Oxidative Stress Index; TAC: Total Antioxidant Capacity; TOS: Total Oxidant Status. *Kruskal-Wallis test.

Table 3. Pairwise comparison of the results between the study groups

	Control vs. I/R	Control vs. I/R + VA	Control vs. I/R + RA	I/R vs. I/R + VA	I/R vs. I/R + RA	I/R + VA vs. I/R + RA
Myofibrillar Edema	<0.001	0.002	0.002	0.005	0.066	0.714
Myocytolysis Focal	0.009	0.009	0.009	>0.99	>0.99	>0.99
Hemorrhage	0.519	0.099	>0.99	0.330	0.519	0.099
PMNL Infiltration	0.537	0.537	0.537	>0.99	>0.99	>0.99
TOS	0.005	0.027	0.001	0.115	0.046	0.014
TAC	0.002	0.009	0.006	0.016	0.016	0.793
OSI (TOS/TAS)	0.002	0.021	0.002	0.027	0.875	0.036
Arylesterase Activity	0.208	0.059	0.115	0.401	0.834	0.345
Lactonase Activity	0.227	0.016	0.318	0.016	0.600	0.021
8-OHdG	0.141	0.916	0.141	0.294	0.834	0.248

*Mann-Whitney U test was used in pairwise comparison.

ence was found between all groups (p>0.05). However, when comparing the lactonase activity between the groups, a significantly higher lactonase activity was found in the I/R + VA group compared to the other three groups (p<0.05). Additionally, the amount of 8-OH deoxyguanosine measured in blood samples did not differ between groups (p>0.05).

The biochemical test results and the comparison between the groups are shown in Table 2. The statistical significance values (p-value) resulting from the pairwise comparisons of the groups with regard to the histopathological evaluation and the biochemical measurements are shown in Table 3.

DISCUSSION

Limb ischemia can be both an emergent pathology and a phase of surgical intervention in cardiovascular surgery. Enzyme activity and antioxidant production decrease in the extremities where ischemia develops or an ischemic state is created. When the ischemic state is eliminated and reperfusion is achieved, ROS are produced and enter the systemic circulation. Many molecules have been used in IRI models to reduce the effects of ROS on end and/or remote organs.[8-11] In our study, we used VA and RA in a lower limb ischemia/reperfusion model to investigate their protective effects on cardiac tissue and oxidative stress. We found that both VA and RA improved antioxidant status. In a pairwise comparison, VA performed better than RA in terms of increasing lactonase activity.

Ischemia and subsequent reperfusion of the ischemic tissue trigger a series of reactions such as the release of ROS, activation of leukocytes, release of cytokines, and complement activation. Released cytokines, especially tumor necrosis factor alpha (TNF-α), lead to leukocyte activation and also disrupt the integrity of the capillary membrane.[12] The activity of sodium-potassium adenosine triphosphatase (Na-K-ATPase) is disturbed by the increasing amount of H2O2 in the environment, which upsets the balance of sodium and potassium ions inside and outside the cell.[13] When the ionic balance between the inside and outside of the cell is disturbed, sodium moves out of the cell, while water moves into the cell from the interstitial area, causing the cell to swell. In our study, it was observed that myofibrillar edema was lowest in the control group and highest in the I/R group. Myofibrillar edema was significantly lower in subjects in the I/R + VA group than in the I/R group. The known antioxidant effects of VA include the removal of ROS from the environment, the induction of reduction reactions, and the inhibition of lipid peroxidation. VA ensures the restoration of enzymatic and non-enzymatic antioxidants in the environment.[14-15] One of the anti-inflammatory effects of VA is that it suppresses TNF-α production.[16] RA also shows anti-inflammatory properties by suppressing the activity and synthesis of TNF-α.[17] Our results show that myofibrillar edema was significantly lower in the I/R + VA and I/R + RA groups, suggesting that VA and RA stabilize membranes against oxidative and inflammatory effects.

The antioxidant and anti-inflammatory effects of foods and plants rich in compounds with phenolic rings have been demonstrated in many studies.[18-21] The VA we used in our study contains one phenolic ring in its component, and RA contains two phenolic rings. In the study conducted by Vinothiya et al. on diabetic and hypertensive rats, it was reported that VA plays a role in the removal of ROS from the environment and increases antioxidant activity.[22] Similarly, there are studies reporting that RA has beneficial effects on TAC in rats with IRI.[6,23] In our study, TAC was higher in the I/R + RA and I/R + VA groups than in the I/R group. When both groups were compared with the I/R group, it was found that both RA and VA increased the antioxidant capacity. However, when the two molecules were compared with each other, no significant difference was found between them in terms of TAC.

The enzyme lactonase, involved in the hydrolysis of lactones, which are cyclic carboxylic acid esters, and the enzyme family of human paraoxonase, which has lactonase and arylesterase activities, has been shown to have antioxidant activity.[24,25] Our study showed that lactonase activity was significantly higher in the I/R + VA group compared to the other groups. When the I/R + RA group was compared with the other groups, no significant difference was found in terms of lactonase activity. No significant difference was found between the groups in terms of arylesterase activity.

8-Hydroxy-2′-deoxyguanosine is formed by hydroxylation of the deoxyguanosine residue in DNA. ROS molecules are known to act as mutagens by triggering this hydroxylation reaction.[26] In our study, the amount of 8-hydroxy-2′-deoxyguanosine was measured in the serum of the subjects and no significant difference was found between the groups. However, the values determined in the control group were closest to those of the I/R + VA group. The results of the I/R + RA group and the I/R group were similar.

Study Limitations

Our study certainly has some limitations that should be acknowledged. Firstly, it is an experimental animal model. This makes it difficult to determine the clinical relevance of animal study results when adapting any compound for therapeutic use. We conducted this study at only one concentration, but different results may be obtained at various concentrations. Secondly, the route of administration of the compounds used in our study was peritoneal, but different results may be obtained with intravenous administration. Third, we examined the effects of the compounds only two hours after reperfusion. It is possible that the pharmacological effects of these compounds vary at different time points after the reperfusion event. Finally, the study was limited to isolated I/R without a thrombotic tendency, and thrombotic metabolism may lead to different results in different study groups with a thrombotic tendency.

CONCLUSION

In summary, VA and RA, which contain phenolic rings, are useful in reducing the effects of ischemia-reperfusion injury on cardiac tissue. Both molecules similarly reduce myofibrillar edema and increase overall antioxidant capacity. In addition, VA increases lactonase activity more than RA. Therefore, we

believe that VA is superior to RA in creating an antioxidant environment.

Ethics Committee Approval: This study was approved by the Trakya University Ethics Committee (Date: 01.09.2022, Decision No: 2021.01.01).

Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept: S.H.; Design: S.H., O.G.; Supervision: S.C.; Resource: S.H.; Materials: S.H., O.G., A.R.; Data collection and/or processing: S.T., B.Ö.; Analysis and/or interpretation: A.R., S.T., B.Ö.; Literature search: A.R., Ü.H.; Writing: A.R., Ü.H., S.H.; Critical reviews: S.H., O.G.

Conflict of Interest: None declared.

Financial Disclosure: The author declared that this study has received no financial support.

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DENEYSEL ÇALIŞMA - *ÖZ*

Vanilik ve rosmarinik asidin kalp dokusu üzerindeki koruyucu etkilerinin karşılaştırılması: Sıçanlarda alt ekstremite iskemi-reperfüzyon modeli

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AMAÇ: İskemi/reperfüzyon hasarı, hem uzun klemp süresi gerektiren elektif prosedürlerde hem de damar tıkanıklığı olan gecikmiş acil vakalarda damar cerrahisinde görülen en zorlu postoperatif durumlardan biridir. İskemi sırasında gelişen enflamatuvar yanıt ve reperfüzyon sırasında artan serbest oksijen radikallerinin beyin, kalp ve böbrekler üzerinde zararlı etkileri vardır. Bu çalışmada, sıçanlarda alt ekstremite iskemi/reperfüzyon modelinde vanilik ve rosmarinik asidin iskemi/reperfüzyon hasarını önlemedeki etkisini karşılaştırmayı amaçladık.

GEREÇ VE YÖNTEM: Ağırlıkları 185-240 g olan, 32 adet dişi Sprague-Dawley ırkı sıçan, rastgele her biri 8 hayvandan oluşan 4 gruba ayrıldı. Grup 1 kontrol, grup 2 iskemi/reperfüzyon (I/R), grup 3 iskemi/reperfüzyon + vanilik asit (I/R + VA) ve grup 4 iskemi/reperfüzyon + rosmarinik asit (I/R +) olarak belirlendi. RA). Kontrol grubu dışındaki tüm gruplarda infrarenal abdominal aorta klemplenerek 60 dakika iskemi ve ardından 120 dakika reperfüzyon uygulandı. Reperfüzyon başlamadan 15 dakika önce Grup 3'e vanilik asit, grup 4'e ise rosmarinik asit intra-abdominal olarak uygulandı. Reperfüzyon fazının sonunda kan örnekleri ve kalp dokuları alınarak sıçanlar sakrifiye edildi. Histopatolojik olarak miyofibriler ödem, miyositoliz, fokal kanama ve kalp dokusunda polimorfonükleer lökosit (PMNL) infiltrasyonu incelendi. Kan örneklerinde total antioksidan kapasite (TAK), total oksidatif durum (TOD), oksidatif stres indeksi (OSİ), 8-OH-deoksiguanozin, laktonaz ve arilesteraz aktivitesi ölçüldü.

BULGULAR: Miyofibriler ödem en çok İ/R grubunda belirgindi ve İ/R + VA ve İ/R + RA gruplarında daha az belirgindi (sırasıyla, p=0.005 ve p=0.066). İskemi/reperfüzyon grupları arasında miyositoliz, fokal kanama ve PMNL infiltrasyonu açısından fark yoktu (p>0.99). Tüm gruplar arasında TOD ve OSİ kontrol grubunda en düşük, TAK ise en yüksekti. TAK, İ/R + VA ve İ/R + RA gruplarında benzer iken, bu iki grupta İ/R grubuna göre anlamlı olarak yüksekti. İ/R + VA grubundaki laktonaz aktivitesi kontrol grubuyla benzer iken İ/R ve İ/R + RA gruplarına göre anlamlı derecede yüksekti.

SONUÇ: Çalışmamız vanilik ve rosmarinik asitin alt ekstremite iskemisi sonrası kalpteki miyofibriler ödemi azalttığını ve TAK'ı arttırdığını göstermektedir. Bununla birlikte, vanilik asit, antioksidan etkisi ile bilinen laktonaz enziminin aktivitesini rosmarinik asite göre daha fazla arttırır.

Anahtar sözcükler: İskemi; kardiyak; laktonaz; reperfüzyon; rosmarinik; vanilik.

Ulus Travma Acil Cerrahi Derg 2024;30(9):619-625 DOI: 10.14744/tjtes.2024.12359