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Activity of Diosmin-Hesperidin in Kidney Ischemia-reperfusion Injury in Rats: Experimental Study

Ratlarda Böbrek İskemi-Reperfüzyon Hasarında Diosmin-Hesperidin'in Etkinliği: Deneysel Çalışma

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Abstract

Objective: We aimed to investigate the activity of diosmin-hesperidin (DAFLON®) in ischemia-reperfusion injury in the rat kidney.
Materials and Methods: Twenty-four Wistar rats were used for the experimental study. Each group comprised of 8 subjects. Sham group (Group 1) was nephrectomized for histopathologic examination and blood samples were obtained for biochemical analysis. Control group (Group 2) was subjected to ischemia for 1 hour and reperfusion for 24 hours, then they were nephrectomized for histopathologic examination and blood samples were taken for biochemical analysis. Treatment group (Group 3) was given 80 mg/kg diosmin-hesperidin combination (DAFLON®) for 10 days and subjected to ischemia for 1-hour and reperfusion for 24 hours, then they were nephrectomized for histopathologic examination and blood samples were obtained for biochemical analysis.
Results: As a result of biochemical analysis and histopathologic examination, more significant results were acquired in regard to other groups. Serum urea values were statistically significant ($p<0.05$). Consistent with the results of the studies previously performed, higher creatinine values were detected in the control and treatment groups. Enzymatic activities of superoxide dismutase and glutathione peroxidase were significantly lower in the treatment group when compared with the control group. When groups were compared based on histopathologic examination findings, cell necrosis and ischemic alterations were statistically significant parameters ($p<0.05$). When all parameters were analysed, values indicating any histopathologic abnormalities were at the lowest level in the treatment group. Histopathologic alterations were most frequently detected in the control group.
Conclusion: Anti-inflammatory activity of diosmin-hesperidin which is used experimentally in the treatment of ischemia-reperfusion injury was evaluated both biochemically and histopathologically. Based on the literature data, it is thought that the damage occurring after ischemia-reperfusion injury can be prevented with diosmin-hesperidin treatment.
Keywords: kidney, ischemia-reperfusion injury, diosmin-hesperidin

Özet

Amaç: Rat böbreğinde iskemi-reperfüzyon hasarında diosmin-hesperidin'in (DAFLON®) etkinliğini deneysel bir çalışma ile araştırmayı amaçladık.
Gereçler ve Yöntemler: 24 adet Wistar rat deneysel çalışma için kullanıldı. Her grup 8 adet denekten oluşmaktaydı. Sham grubu (Grup 1) histopatolojik inceleme için nefrektomi ve biyokimyasal analiz için kan alınan gruptu. Kontrol grubu (Grup 2) 1 saat iskemi ve 24 saat reperfüzyon yapılıp sonrasında histopatolojik inceleme için nefrektomi ve biyokimyasal analiz izin kan alınan gruptu. Tedavi grubu (Grup 3) ise 10 gün boyunca 80mg/kg/gün dozunda diosmin hesperidin (DAFLON®) verildikten sonra 1 saat iskemi ve 24 saat reperfüzyon yapılıp sonrasında histopatolojik inceleme için nefrektomi ve biyokimyasal analiz için kan alınan gruptu.
Bulgular: Yapılan biyokimyasal analizler ve histopatolojik değerlendirmeler sonucunda tedavi grubunda diğer gruplara göre daha anlamlı sonuçlar elde edildi. Serum üre değerleri istatistiksel olarak anlamlıydı ($p<0.05$). Diğer biyokimyasal parametrelerden kreatinin değeri ise yapılan çalışmalarla doğru orantılı olarak kontrol grubu ve tedavi grubunda yüksek bulundu. Süperoksit dismutaz ve Glutatyon peroksidaz enzim aktiviteleri de tedavi grubunda kontrol grubuyla karşılaştırıldığında anlamlı derecede düşük bulundu. Histopatolojik değerlendirmeler sonucunda da gruplar karşılaştırıldığında hücre nekrozu ve iskemik değişiklikler istatistiksel olarak anlamlıydı ($p<0.05$). Bütün parametreler incelendiğinde histopatolojik bozukluğu gösteren değerler tedavi grubunda en alt düzeydeydi. En fazla ise kontrol grubunda değişimler mevcuttu.
Sonuç: İskemi reperfüzyon hasarında deneysel olarak kullanılan diosmin-hesperidin etkinliği hem biyokimyasal hem de histopatolojik olarak değerlendirildi. Literatür gözden geçirildiğinde, iskemi- reperfüzyon hasarı sonrası diosmin-hesperidin tedavisi ile oluşan hasarın engellenebileceği düşünülmektedir.
Anahtar kelimeler: böbrek, iskemi-reperfüzyon hasarı, diosmin-hesperidin

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Introduction

With the higher prevalence of surgical interventions that reduce renal blood flow such as transplantation, trauma, anatrophic nephrolithotomy, nephron-sparing surgery, and renal artery surgery ischemia-reperfusion injury has been more frequently cited in the literature. In ischemia, oxygen required to maintain aerobic metabolism is not supplied to the living tissue. Recovery of normal blood flow after a period of ischemia is called reperfusion. Since the self-control of the metabolism of the oxygen entering the atmosphere with reperfusion of the organ that remains ischemic for a while deteriorates, free oxygen radicals (FORs) with their toxic effects become manifest and cause ischemia-reperfusion injury in the tissue. Along with emerging FORs, activation of various proteases and phospholipase A2 by calcium entering the cell during ischemia is also a response to ischemia-reperfusion injury [1-5].

The response that organs give to experimental ischemia-reperfusion injury was very well specified in the rats and rats were preferred as experimental animals in most of the literature studies [2-6]. During ischemia, the tissue is damaged by asphyxiation and when the normal blood flow is retrieved, tissue damage aggravates greatly as a result of a series of events caused by the oxygen entering the atmosphere [7,8].

Mitochondrial electron transport chain reactions, inhibition of arachidonic acid metabolites, increase in intracellular calcium levels, xanthine oxidase system, iron ion, etc. involve in the production of FORs which induce ischemia-reperfusion injury in the kidney tissue. These factors affect each other sequentially and disrupt cell functions, increase membrane destruction, and result in the production of endogenous toxins [9-13].

The only way of treatment in ischemic kidneys is to increase the renal blood flow through reperfusion. Yet in that case reperfusion damage inevitably occurs. In such a case, since the increase of blood supply is inevitable, it is necessary to look for solutions to prevent reperfusion damage. Many agents such as vitamin E, melatonin, phospholipase type 3 enzyme inhibitors (amrinone, olprinone), adenosine, n-acetyl cysteine, nitric oxide (NO), calcium channel blockers, mycophenolate mofetil have been used in order to prevent or alleviate renal ischemia-reperfusion injury associated with various etiologic factors [10,11].

Diosmin-hesperidin is produced by the purification of flavonoid extracts of a plant found in the nature. Daflon is a phlebotonic and vasculoprotector agent that is comprised of 90% diosmin and 10% hesperidin. Hesperidin reinforces the activity of diosmin, improves wound recovery by acting against inflammatory mediators and protects microcirculation via decreasing blood viscosity [14].

Diosmin-hesperidin has also shown anti-inflammatory effects through many mechanisms of action in our study. Many studies have been conducted on significant ischemia-reperfusion injury preventing effects of diosmin-hesperidin in multiple organs such as heart, brain muscle tissue, and peritoneum [14,15].

Diosmin-hesperidin is an important antioxidant drug combination. Leukocyte aggregation is important in ischemia-reperfusion injury and damage can be prevented with diosmin-hesperidin at daily oral doses of 500 mg. Also, this drug combination reduces the amount of H2O2 released from leukocytes by suppressing activity of myeloperoxidase (MPO). A decrease in the MPO activity can explain the decrease in H2O2 in the group that received diosmin-hesperidin at doses protecting against oxidative stress associated with glutathione (GSH). Diosmin-hesperidin given at doses protecting against oxidative stress associated with GSH guards the escape of macromolecules from the microvascular structures. According to histopathological data, diosmin-hesperidin prevents infiltration of leukocytes into the perivascular area. Although it does not totally prevent leukocyte infiltration, a significant reduction in leukocyte accumulation in the perivascular area in the kidney was observed [16].

We have aimed to experimentally investigate the activity of diosmin-hesperidin, which acts against inflammatory mediators in ischemia-reperfusion injury and protects microcirculation by decreasing blood viscosity in cases with renal ischemia-reperfusion injury.

Materials and Methods

Twenty-four healthy adult Wistar rats weighing between 300-380 gr (average 340 gr) were used in the study. Experimental animals were tracked by keeping them at normal room temperature and in separate cages during preoperative and postoperative periods. Anesthesia was provided with the combination of ketamine IM (40 mg/kg, Ketalar 50 mg/cc, Parke-Davis) and xylazin HCI IV (10 mg/kg, Rompun, 23-32 mg/cc, Bayer). According to experimental protocol maintenance of anesthesia was provided with the same doses. Ethics committee approval for the study was acquired from the Ethics Committee of Ankara Training and Research Hospital (Date: 18.02.2009, Decision No: 2347).

In this study, rats were separated into three groups of 8 subjects. Experimental animals were tracked for 10 days by being kept at normal room temperature and in separate cages during preoperative and postoperative periods. Groups 1 and 2 were followed up without giving any treatment. Daily doses of 80 mg/kg diosmin-hesperidin (DAFLON®) were given to Group 3 subjects for 10 days by crushing film-coated tablets and adding them in their drinking water. At the end of the 10th day, only Group 1 rats underwent nephrectomy through laparotomy incision. In Groups 2 and 3, ischemia was induced in the renal artery for 60 minutes. After 24 hours, for the subjects in each group, before the procedure, serum samples were collected for the measurement of glutathione peroxidase, superoxide dismutase and urea creatinine. Then nephrectomy specimens in 10% formaldehyde solution were sent for histopathologic evaluation **(Figure 1).**

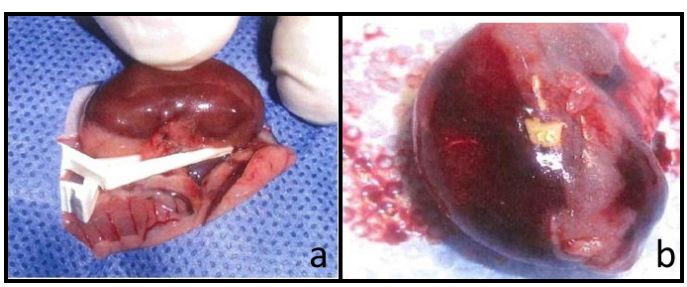

Figure 1a. Right kidney on which warm ischemia model with microvascular clamp constituted (Groups 2 and 3) **1b.** Nephrectomy performed right kidney after ischemia-reperfusion injury

After tissue samples in 10% formaldehyde were embedded in paraffin blocks and stained with hematoxylin-eosin, all preparations were examined under light microscope by a pathologist blinded to the study protocol. A semiquantitative scale defined by Paller et al. who evaluated, and scored the alterations in acute renal insufficiency was used [1].

Modified scoring system defined by Paller et al:
1- Chronic inflammation (0, +, ++)
2- Tubular epithelial cell flattening (0, +, ++)
3- Cytoplasmic vacuoalization (0, +, ++)
4- Cell necrosis and ischemic changes (0, +, ++)
5- Tubular lumen obstruction (0, +, ++)

Statistical Analysis

Data analysis was performed using the SPSS 15 package program. While descriptive statistics were shown as mean ± standard deviation for blood creatinine values, alterations in histopathologic results and blood parameters were shown as average (minimum-maximum) values. One-Way Anova analysis was used in the comparisons of blood parameters both within and between groups. T-test with Bonferroni correction was used to investigate whether there is a significant alteration in the groups in regard to serum urea creatinine levels. Percent changes between groups in serum urea creatinine levels within 24 hours were calculated, and intergroup comparisons were performed. The statistical significance of histopathologic scores between groups was analyzed using the Kruskal-Wallis test. The results were accepted as statistically significant at $p<0.05$.

Results

Serum Urea Values

Average serum urea values were significantly higher in the control (Group 2) and treatment (Group 3) groups compared to the sham group (Group 1). The highest value was found in the control group. A statistically significant difference was detected between the groups ($p<0.05$) **(Figure 2).**

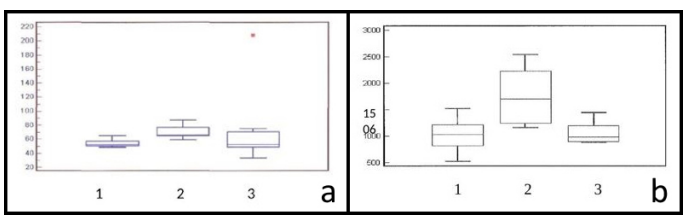

Figure 2a. Range of urea values between groups **2b.** Range of creatinine values between groups

Serum Creatinine Values

Average serum creatinine values were higher in the control and the treatment groups compared to the sham group. There was no statistically significant difference between the groups in this regard ($p>0.05$) **(Figure 2).**

Superoxide Dismutase Enzyme Values

Superoxide dismutase enzyme values were higher in the control group compared to the other groups. The sham group and treatment group had approximately the same values. There was no statistically significant difference between the groups in this regard ($p>0.05$).

Glutathione Peroxidase Enzyme Values

Highest glutathione peroxidase enzyme values were found in the control group. The sham group and treatment group had approximately the same values. There was no statistically significant difference between the groups in this regard ($p>0.05$).

Results of Histopathological Evaluation

According to histopathological classification defined by Paller et al., a total of 100 cortical tubules from 10 different microscopic field of views were evaluated in the kidney preparation of each experimental animal. An average score for each case was stated by averaging the scores. From the average scores of 8 rats in each group, the general average and standard deviation were calculated.

Histopathological evaluation revealed the presence of significant intergroup differences. Especially cell necrosis, tubular lumen obstruction, chronic inflammation, cytoplasmic vacuolization, and epithelial cell flattening were more common in the control and treatment groups when compared with the sham group. The most distinct histopathological alterations were cell necrosis and inflammation which were observed significantly less frequently in the treatment group relative to the control group **(Figure 3).**

There were histopathological differences between the control group and the treatment group. Histopathological alterations which are the indicator of kidney insufficiency were more common in the control group. The frequency of histopathological alterations in the treatment group was nearly close to the sham group. The most distinct alteration was cell necrosis and ischemic changes were less common in the treatment group. Cell necrosis and ischemic alterations were statistically significant findings ($p<0.05$).

Histopathological alterations indicating all reperfusion injuries were observed more commonly in the control group. In the treatment group, severe histopathological alterations were observed less frequently compared to the control group. The histopathological alterations caused by kidney reperfusion injury in the treatment group were observed significantly less frequently compared to the control group. The most distinct alteration was cell necrosis and ischemic changes were less common in the treatment group ($p<0.05$) **(Table 1).**

As a result of histopathological evaluations, the most distinct alteration was found in the treatment group. As a critically important finding cell necrosis and ischemic alterations were significantly different between the treatment, and control groups. There were partial cell necrosis and ischemic alterations in the treatment group, while cell necrosis and ischemic alterations were more frequently seen in the control group with a statistically significant intergroup difference ($p<0.05$) **(Figure 3).**

Discussion

There is no need for specifying a specific ischemia time for the revelation of the effects of experimental ischemia-reperfusion injury. In experiments performed with rat kidneys, Williams et al. [5] indicated that reperfusion injury emerged after ischemia lasting for 45 minutes, while Paller et al. [1]

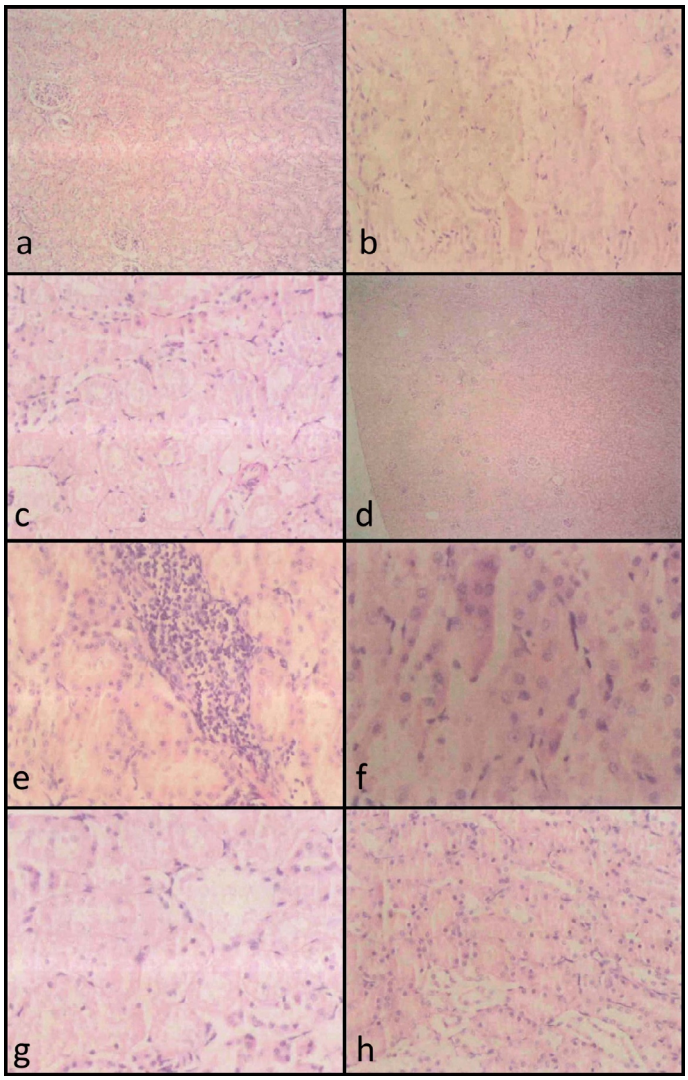

Figure 3a. Histopathological analysis of the kidney, all histopathological sections were dyed with hematoxylin-eosin (HEx100). Necrosis in the tubules of the sham group and cell casts (debris) in the tubule were followed insignificantly. **3b.** Ischemia-reperfusion + Daflon (80 mg/kg/day) performed treatment group. It was observed that in necrosis in the tubuler cells decreased at the $p<0.05$ level compared to the control group, and there was a significant decrease in the intratubular caste deposition at the $p<0.05$ level. **3c.** Distinct tubular cell necrosis and intratubular caste deposition were noticed in the control group. In the treatment group, these changes were less than in the control group and were in the form of focal alterations. **3d.** Ischemic alterations were noticed in the control group. In the treatment group, these changes were less than in the control group and could be observed focally. **3e.** Alterations related to chronic inflammation were noticed in the control group. In the treatment group, these changes were less than in the control group and were focal. **3f.** Alterations related to tubular lumen obstruction were noticed in the control group. In the treatment group, these changes were less than in the control group. **3g.** Alterations related to the flattening in the tubular luminal epithelium were noticed in the control group. In the treatment group, these changes were less than in the control group and were focal. 3h. Alterations related to the cytoplasmic vacuolization were noticed in the control group. In the treatment group, these changes were less than in the control group and were at a focal level.

Table 1. Histopathological data

	Group1		Group 2		Group 3		p
Chronic inflammation	n	%	n	%	n	%	
	7	87.5	3	37.5	5	62.5	0.253
	1	12.5	4	50	3	37.5	
	0	0	1	12.5	0	0	
Tubular epithelial cell flattening	n	%	n	%	n	%	
	6	85.7	4	50	5	62.5	0.261
	1	14.3	2	25	3	37.5	
	0	0	2	25	0	0	
Cytoplasmic vacuolization	n	%	n	%	n	%	
	6	75	1	12.5	3	37.5	0.126
	2	25	5	62.5	3	37.5	
	0	0	2	25	2	25	
Cell Necrosis and ishcemic changes	n	%	n	%	n	%	
	8	100	0	0	5	62.5	0.000
	0	0	2	25	3	37.5	
	0	0	6	75	0	0	
Tubular lumen obstruction	n	%	n	%	n	%	
	8	100	3	37.5	5	62.5	0.127
	0	0	3	37.5	2	25	
	0	0	2	25	1	12.5	

stated that it emerged after ischemia persisting for 60 minutes. In this study, renal ischemia applied for 60 minutes was preferred in consistent with literature data. It has been reported that renal ischemia-reperfusion injury becomes manifest at the earliest within 4 hours based on the analyzes of tissue samples and serum analytes and reached its peak at 24 hours [9]. As we desired to investigate the effects of renal ischemia-reperfusion injury clearly in our study, other procedures were also initiated 24 hours after reperfusion.

In our study; we observed that in the model we formed by applying ischemia for 60 minutes and reperfusion for 24 hours, the levels of plasma urea and creatinine which are indicators of renal glomerular insufficiency in the treatment and the control groups increased. While serum urea values were statistically significantly higher ($p<0.05$), creatinine values were not. However, their median average values were significantly increased. Scarce number of data, uncertainties about the dosages and time periods specified for the treatment were thought to be the reasons for not obtaining statistically significant results .

A significant reduction in activities of renal antioxidant enzymes and histopathological manifestations suggestive of

necrosis was observed. Low antioxidant enzyme activity is an indicator of a mild ischemia-reperfusion injury. Enzyme activities were at the highest level in the control group. Because this group was exposed most often to the adverse effects of ischemia-reperfusion injury compared to the other groups. In the sham and treatment groups, enzyme activities were almost at the same level and quite low compared to the control group. As a result of this finding, it can interpreted that the treatment group was least affected by ischemia-reperfusion injury.

When studies were reviewed, there was no specific dosage used for the prevention of renal ischemia-reperfusion injury [1,5]. An average dose used in our study was determined based on the studies conducted on other organs.

Histopathologically there was a statistically significant decrease, especially in cell necrosis and ischemic alterations ($p<0.05$). The scoring system defined by Paller et al. [1] and used in acute kidney insufficiency was modified and applied in our study. We detected that the treatment group was more effective in improving all parameters taken into consideration and less damaging to the cell relative to the control group. Our findings were consistent with those of many studies investigating therapeutic activity of diosmin-hesperidin in muscle tissue, brain tissue, peritoneum, and heart tissue with ensuing histopathologically significant results [14,15].

Dobashi et al. applied renal ischemia (30, 60, 90 min.) and reperfusion (2, 24, 72, 120 hours) for different time periods, and noticed a significant decrease in superoxide dismutase and glutathione peroxidase activities and increase at the level of lipid peroxidation in the group in which they applied ischemia for 60 minutes and reperfusion for 24 hours [15]. Also in the studies where ischemia and reperfusion were applied for different durations, decrease in glutathione levels whereas increase in peroxidation and plasma urea and creatinine levels were indicated. Our findings were consistent with the results of previous studies [15,17].

Many studies cited in the literature have shown the favborable effect of diosmin-hesperidin on alleviating or preventing ischemia-reperfusion injury [14,15]. In our study, diosmin-hesperidin at a dose of 500 mg that prevented the development of ischemia-reperfusion injury in other organs was also found to be effective in preventing renal ischemia-reperfusion injury.

Diosmin-hesperidin is a medication used in venous insufficiency of extremities and venous system diseases such as hemorrhoids. Its venous tone enhancing, capillary resistance, and capillary permeability regulating, and lymphatic drainage enhancing effects are known. Besides, its free radical scavenging and leukocyte adherence-reducing effects have been shown. Duchene-Marullaz et al. demonstrated that it significantly reduced free radical activity, chemotactic reaction, and free radical activity induced by leucocyte phagocytosis consistent with our findings [18].

Stucker et al. conducted a microvascular permeability study using fibrils of cremaster muscle, and indicated that the treatment with diosmin-hesperidin also significantly reduced the residue induced by ischemia and bradykinin in vascular permeability [19]. In in vitro and in vivo studies, Di Peri and Auteri demonstrated that diosmin-hesperidin reduced the activity of the complement system, and this mechanism of action was considered to constitute one of the anti-inflammatory effects of this drug therapy which also contributes favorably to the ulcer recovery [20].

Schoab et al. conducted a study on patients with chronic venous insufficiency using diosmin-hesperidin, and showed that it increased wound recovery by reducing oscillations in endothelial VCAM1 and ICAM1 expressions, preventing leukocyte adhesion, activation, and migration which consequently shortening the recovery time in patients with venous insufficiency [21]. Coloridge-Smith et al. conducted a meta-analysis on patients with venous ulcers developed as a result of chronic venous insufficiency, and showed that administering diosmin-hesperidin in addition to conventional treatment was effective in the rapid reduction of ulcer-related symptoms and sizes of ulcers compared to conventional treatment only [22].

Hasanoğlu et al., showed that diosmin-hesperidin was systematically and topically effective in infected skin injuries and improved wound recovery [23,24]. Pecking et al. investigated the effects of diosmin-hesperidin in upper extremity lymphedema developed after breast cancer surgery, and showed that diosmin-hesperidin was an effective treatment of lymphedema by increasing lymphatic flow in lymphedema, significantly reducing intralymphatic pressure and at the same time increasing the return of interstitial fluid to the capillaries [16]. Hasanoğlu et al. used diosmin-hesperidin systematically and topically in infected second-degree burns and showed that wound recovery was faster and better in patients in whom diosmin-hesperidin was used [24].

Many above-mentioned studies have indicated that diosmin-hesperidin was effective in preventing inflammatory effects and injury caused by free oxygen radicals. Therefore, frequent use of diosmin-hesperidin at the experimental level as an anti-inflammatory drug in ischemia-reperfusion injury conveys extreme importance as has been always observed in previously performed studies. Our study has shown that diosmin-hesperidin exerted anti-inflammatory effects that prevented development of ischemia-reperfusion injury.

Conclusion

In this study, the effect of diosmin-hesperidin on renal ischemia-reperfusion injury and whether this drug treatment can be used before development of ischemia-reperfusion injury were investigated. The effects of diosmin-hesperidin on ischemia-reperfusion injury were histopathologically and biochemically evaluated. As a result of histopathological and biochemical evaluations, favorable results of diosmin-hesperidin on the prevention of ischemia-reperfusion injury were obtained. When the literature is reviewed, it has been concluded that the damage caused by ischemia-reperfusion injury can be prevented with diosmin-hesperidin treatment.

Ethics Committee Approval: Ethics committee approval for the study was acquired from the Ethics Committee of Ankara Training and Research Hospital (Date: 18.02.2009, Decision No: 2347).

Informed Consent: An informed consent was obtained from all the patients.

Publication: The results of the study were not published in full or in part in form of abstracts.

Peer-review: Externally and internally peer-reviewed.
Authorship Contributions: Any contribution was not made by any individual not listed as an author. Concept – K.Y., E.E.; Design – K.Y., E.E.; Supervision – K.Y., E.E.; Resources – K.Y., E.E.; Materials – K.Y., E.E.; Data Collection and/or Processing – K.Y., E.E.; Analysis and/or Interpretation – K.Y., E.E.; Literature Search – K.Y., E.E.; Writing Manuscript – K.Y., E.E.; Critical Review – K.Y., E.E.
Conflict of Interest: The authors declare that they have no conflicts of interest.
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