The effect of 3′,4′-dihydroxyflavonol on plasma oxidant and antioxidant systems in testis ischemia-reperfusion injury in rats

Aysel Duman 1, Rasim Mogulkoc 1,*, Abdulkerim Kasim Baltaci 1, Abdullah Sivrikaya 2

1Selcuk University, Medical Faculty, Department of Physiology, Turkey
2Selcuk University, Medical Faculty, Department of Biochemistry, Turkey

*corresponding author e-mail address: rasimmogulkoc@yahoo.com

ABSTRACT
The purpose of the present study was to determine the effect of 3′,4′-dihydroxyflavonol (DiOHF) on plasma lipid peroxidation in experimental testicular torsion-detorsion. The study involved 60 Wistar albino type male rats weighing 250-260 gr. The experimental groups were formed as follows: 1. Control; 2. Sham-control; 3. 720°-4 hours torsion; 4. 720°-4 hours torsion + 4 hours detorsion; 5. 720°-4 hours torsion + DiOHF; 6. 720°-4 hours torsion + DiOHF + 4 hours detorsion; 7. 720°-4 hours torsion + 24 hours detorsion; 8. 720°-4 hours torsion + DiOHF + 24 hours detorsion. The animals in the experimental groups were anesthetized after the procedures and their blood samples were taken to determine the levels of plasma glutathione peroxidase (GPx), nitric oxide (NO), malondialdehyde (MDA), and erythrocyte glutathione (GSH). Among the study groups, group 5 was found to have the highest plasma glutathione peroxidase values (p<0.001). Groups 3 and 4, which were torsion and detorsion groups, had the lowest plasma GPx values (p<0.001). Plasma NO values were found to be higher in groups 3 and 4 than all other groups (p<0.001). Groups 3, 4, and 7 had the highest plasma MDA levels (p<0.001). Erythrocyte GSH levels in groups 5 and 7 were significantly higher than the levels in other groups (p<0.001). The results of the study indicate that lipid peroxidation that increases in plasma during testis ischemia-reperfusion injury in rats is prevented by intra-peritoneal DiOHF administration.

Keywords: testis ischemia-reperfusion; 3′,4′-dihydroxyflavonol; rat.

1. INTRODUCTION
A decrease in the blood supply to a tissue as a result of the obstruction of the blood vessels feeding the concerned tissue due to a clot or a mechanical cause is called ischemia [1]. Restoration of the blood supply to the tissue through drugs or mechanical interventions is called reperfusion. Ischemia-reperfusion (IR) injury is the cellular damage that occurs after the organ which suffered hypoxia is re-oxygenated. The severity of the reperfusion injury depends on the re-oxygenation after reperfusion, rather than the accumulated effects of the damage that occurred during ischemia. Oxidative stress created by free radicals is known as the cause of organ injury [2]. Paradoxically, reperfusion of the ischemic tissue inflicts more severe injury to the tissue than that caused by ischemia alone [3]. There are many mechanisms involved in the injury that is incurred in the reperfusion period including, first and foremost, the free radical derivatives that are formed rapidly when molecular oxygen enters into the cell. The cellular structures that are most susceptible to reperfusion injury are membrane lipids, proteins, nucleic acids, and deoxyribonucleic acid molecules [4].

Insufficient oxygen supply to meet metabolic needs, depletion of cellular energy reserves, and accumulation of toxic metabolites during ischemia lead to germ cell death [5]. In the reperfusion stage, both reactive oxygen radicals (ROS) and reactive nitrogen derivatives (RNS) are significantly elevated [6]. These free radicals cause lipid peroxidation in the mitochondria and cell membrane, which in turn leads to increased membrane permeability or impaired membrane integrity [6]. Flavonoid-type compounds were reported to have anti-oxidative [7].

The purpose of the present study is to determine the effect of 3′, 4′-dihydroxyflavonol, a synthetic flavonoid which was established to have a protective effect in heart and brain ischemia in previous studies, on plasma glutathione peroxidase (GPx), nitric oxide (NO), malondialdehyde (MDA), and erythrocyte glutathione (GSH) levels in experimental testicular torsion-detorsion.

2. EXPERIMENTAL SECTION
This study was conducted at the Selcuk University Experimental Medicine Research and Application Center after obtaining the approval of the ethics committee. The study included 60 Wistar albino type male rats weighing 250 to 260 gr. All the rats were given feed and water in temperature- and light-controlled rooms. Surgical procedures were carried out after the rats were anesthetized with intramuscular ketamine (60 mg/kg, Eczacibasi) and Xylasine (Rompun, Bayer) (5 mg/kg).

1. General control (n=6): The group in which unilateral orchietomy was performed under anesthesia without any other procedure.
2. Sham control (n=6): The animals in this group were put under general anesthesia (Ketamine + Rompun) and then their testicular area was surgically removed. Additionally, the animals were given an intraperitoneal vehicle of DiOHF.
3. 720°-4 hours torsion (n=8): After the animals in this group were put under general anesthesia, they received 720° torsion for 4 hours on their right testis.

4. 720°- 4 hours torsion + 4 hours detorsion (n=8): After the animals in this group were put under general anesthesia, they received 720° torsion for 4 hours on their right testis. This was followed by 4 hours of detorsion.

5. 720°- 4 hours torsion + DiOHF (n=8): The animals were put under general anesthesia. Then their testis was subjected to 720° torsion for 4 hours. An i.p. DiOHF injection of 30 mg/kg was given on minute 30 of torsion.

6. 720°-4 hours torsion + DiOHF + 4 hours detorsion (n=8): The animals in this group were put under general anesthesia and then their testis was subjected to 720° torsion for 4 hours. After the torsion period, they were given a 30 mg/kg i.p. DiOHF injection. This was followed by 4 hours of detorsion.

7. 720°- 4 hours torsion + 24 hours detorsion (n=8): After being put under general anesthesia, the animals in this group received 720° torsion for 4 hours on their right testis. The torsion period was followed by 24 hours of detorsion.

8. 720°- 4 hours torsion + DiOHF + 24 hours detorsion (n=8): The animals in this study group were put under general anesthesia and then received 720° torsion for 4 hours on their right testis. After the torsion period ended, they were injected with 30 mg/kg i.p. DiOHF and then received 24 hours of detorsion.

When the experimental procedures were completed, all the animals in the study groups were sacrificed under anesthesia and their blood and tissue samples were collected.

**Dihydroxy Flavonoid Injection.** After being dissolved in 4 ml dimethyl sulfoxide, DiOHF (Indofine Chemical Co. USA) was added polyethylene glycol and water to obtain a total volume of 200 ml. The solution was injected into the experimental animals at a dose of 30 mg/kg through the intraperitoneal route [7].

**Collection of blood.** Blood samples of 3 to 4ml were drawn from the anesthetized animals by cardiac puncture and put into EDTA-containing tubes. Erythrocyte GSH levels in the samples were measured immediately, while plasma samples were stored at -80°C until the time of analysis.

**Blood analyses**

**Glutathione peroxidase (GPx) analysis.** Glutathione peroxidase was analyzed with Cayman brand commercial kits (Catalogue No: 703102) according to the colorimetric method. GPx values were expressed as nmol/min/ml.

**Measurement of nitric oxide (NO) levels.** NO levels were quantified with Cayman brand commercial kits (Catalogue No: 780001) following colorimetric method. Nitric oxide levels were presented as µM.

**Measurement of plasma malondialdehyde (MDA) levels.** Plasma MDA levels were determined using Cayman brand commercial kits (Catalogue No: 705002) according to ELISA colorimetric method. The results were presented as nmol/ml.

**Erythrocyte glutathione (GSH) analysis.** Erythrocyte GSH levels were measured using the Elman method. Erythrocyte GSH values were expressed as mg/dl.

**Statistical Evaluations.** Statistical evaluation of the data was conducted using the SPSS statistics software. The results were presented as mean±standard deviation. Kruskal Wallis variance analysis was used in the comparisons between groups. Mann Whitney U test was employed for p<0.05 level. Level of statistical significance was established at p<0.05.

**3. RESULTS SECTION**

Plasma glutathione levels were found to be 5.54±0.66 in the control group, 6.81±0.43 in the sham-control group, 2.99±0.50 in the 720°- 4 hours torsion group, 4.19±1.80 in the 720°-4 hours torsion + 4 hours detorsion group, 10.23±2.12 in the 720°C-4 hours torsion + DiOHF group, 7.86±1.13 in the 720°C-4 hours torsion + DiOHF + 4 hours detorsion group, 5.06±0.72 in the 720°C-4 hours torsion + 24 hours detorsion group, and 9.01±2.08 nmol/min/ml in the 720°C-4 hours torsion + DiOHF + 24 hours detorsion group. The examination of plasma glutathione peroxidase values in the study groups revealed that group 5 (720°-4 hours torsion + DiOHF) had the highest plasma glutathione peroxidase values (p<0.001). Groups 3 and 4, torsion and detorsion groups, on the other hand, had the lowest plasma GPx values (p<0.001). GPx levels in groups 6 and 8 were higher than those in other groups but lower than the levels in group 5 (p<0.001). Groups 1, 2, and 7 had higher plasma GPx levels than groups 3 and 4 (p<0.001) (Figure 2).

**Fig. 1.** *P<0.001 (a>b>c>d). Group 5 has the highest plasma GPx levels compared to other groups.

**Groups:** 1. Control; 2. Sham-control; 3. 720°-4 hours torsion; 4. 720°-4 hours torsion + 4 hours detorsion; 5. 720°-4 hours torsion + DiOHF; 6. 720°-4 hours torsion + DiOHF + 4 hours detorsion; 7. 720°-4 hours torsion + 24 hours detorsion; 8. 720°-4 hours torsion + DiOHF + 24 hours detorsion.

Plasma MDA levels were found to be 6.49±0.66, 6.74±0.27, 9.37±1.82, 9.03±1.95, 6.40±1.29, 7.61±0.86, 9.42±0.83, and (p<0.001). Groups 1, 2, and 8 had the lowest NO levels (p<0.001). (Figure 2).
The highest plasma values of this parameter were established in groups 3, 4, and 7 (p<0.001). Plasma MDA levels in group 6 were lower than the levels in groups 3, 4, and 7, but higher than those in other groups (p<0.001). (Figure 3).

**Fig. 2.** *P<0.001 (a>b>c). Groups 3 and 4 have the highest plasma NO levels compared to other groups. Groups: 1. Control; 2. Sham-control; 3. 720°-4 hours torsion; 4. 720°-4 hours torsion + 4 hours detorsion; 5. 720°-4 hours torsion + DiOHF; 6. 720°-4 hours torsion + DiOHF + 4 hours detorsion; 7. 720°-4 hours torsion + 24 hours detorsion; 8. 720°-4 hours torsion + DiOHF + 24 hours detorsion.

Blood GSH levels of the study groups were 27.97±5.45, 26.47±4.93, 30.66±4.77, 27.93±4.30, 49.54±11.62, 34.57±7.79, 54.44±9.50, and 41.69±6.61 mg/dl, respectively. When erythrocyte GSH levels were examined, groups 5 and 7 were found to have higher erythrocyte GSH levels than other groups (p<0.01). Similarly, this parameter was higher in groups 6 and 8 in comparison to other groups (p<0.01). (Figure 3).

**Fig. 3.** *P<0.001 (a>b>c). Groups 3, 4 and 7 have the highest plasma MDA levels compared to other groups. Groups: 1. Control; 2. Sham-control; 3. 720°-4 hours torsion; 4. 720°-4 hours torsion + 4 hours detorsion; 5. 720°-4 hours torsion + DiOHF; 6. 720°-4 hours torsion + DiOHF + 4 hours detorsion; 7. 720°-4 hours torsion + 24 hours detorsion; 8. 720°-4 hours torsion + DiOHF + 24 hours detorsion.

In the present study, we first aimed to induce ischemia in the unilateral testes of experimental animals. In order to do that, after putting the animals under general anesthesia, we rotated their right testis 720° clockwise and fixed it to the scrotum tissue to keep it ischemic for 4 hours. Next, the same ischemia procedure was accompanied by the administration of 3,4-dihydroxyflavonol which is a synthetic flavonoid whose potency as a tissue protector has been shown in various ischemia-reperfusion studies [10,11]. Thus, the effect of 3,4-dihydroxyflavonol on lipid peroxidation caused by torsion and different reperfusion periods in the testis was examined.

One of the main oxidants against stress in the erythrocytes, GPxs serves important functions in the phagocytes [12]. Ischemia and reperfusion studies present different results about how GPxs is affected. In their experimental myocardial ischemia study, Selvaraj and colleagues [13] reported reduced GPx values in the heart tissue of rats. However, in another study, mesenteric ischemia-reperfusion injury was found to increase GPx values in the intestinal, hepatic, renal, and pulmonary tissues [14]. Elevated glutathione peroxidase levels were established in unilateral testis ischemia-reperfusion injury [15]. In the study by Guan et al. [16]. In our study, GPxs levels after 4 hours of testis ischemia were found lower than the levels in the control group. The same decrease was evident when 4 hours of torsion was followed by 4 hours of detorsion (group 4). However, dihydroxyflavonol administration together with either torsion or torsion and detorsion was found to significantly elevate plasma GPx values, when compared to torsion and control groups. Previous studies already demonstrated that flavonoids were potent antioxidants with tissue protective effects in different organs [6,17]. Differently, from other studies, we used DiOHF, a synthetic flavonoid, in ischemia-reperfusion injury in testis tissue. Use of DiOHF markedly elevated plasma GPx levels in both ischemia and reperfusion and these results are consistent with the results of different research teams obtained with flavonoids in various organs. Thus, our findings regarding DiOHF use in the testes are supported by the results of other researchers.

**Fig. 4.** *P<0.001 (a>b>c). Groups 5 and 7 have the highest plasma erythrocyte GSH levels compared to other groups. Groups: 1. Control; 2. Sham-control; 3. 720°-4 hours torsion; 4. 720°-4 hours torsion + 4 hours detorsion; 5. 720°-4 hours torsion + DiOHF; 6. 720°-4 hours torsion + DiOHF + 4 hours detorsion; 7. 720°-4 hours torsion + 24 hours detorsion; 8. 720°-4 hours torsion + DiOHF + 24 hours detorsion.
In another part of our study, we examined how ischemia-reperfusion and ischemia-reperfusion + DiOHF in testis tissue affected plasma nitric oxide levels. We found that this parameter increased significantly, relative to the control group, after 4-hour ischemia. Administration of 30 mg/kg intraperitoneal DiOHF to ischemia and ischemia and reperfusion groups markedly reduced plasma nitric oxide values which were elevated by ischemia and reperfusion. Results of the studies about the interaction between nitric oxide and free oxygen radicals and its antioxidant properties are inconsistent. As it binds superoxide, NO is believed to be a protective factor that scavenges free radicals. It was reported that intense reactive oxygen species that were released at the onset of reperfusion led to cell injury and tissue death [17]. However, it was also argued that NO released during reperfusion had a protective effect on preconditioning mechanisms (Wang et al., 2011). In fact, it was suggested that nitric oxide had different effects in different I/R injuries [17]. Conversely, peroxynitrite (ONOO−), a product of the reaction between superoxide and NO, is considered a potent oxidant with a long half-life [18]. There are a number of studies about the changes in NO levels during testicular ischemia-reperfusion [19,20]. In a study by Yildiz et al., [21], the right testis was subjected to 720° clockwise torsion for 2 hours and then to detorsion for another 2 hours. Ozokutan et al., [22] showed that suppression of NO synthesis in rats significantly ameliorated the damage inflicted by I/R injury, while increased NO production due to L-arginine exacerbated tissue damage resulting from I/R. Besides, nitric oxide levels measured after detorsion were found higher than the levels measured by torsion. Increased NO levels resulting from torsion and detorsion in our study are parallel to the results of the studies which established an ischemia-associated increase in NO levels. In the present study, DiOHF which was already shown to be a potent antioxidant in cardiac and cerebral ischemia studies was used with torsion and detorsion. DiOHF used with both torsion and detorsion brought about a marked decrease in plasma nitric oxide values. Thus, it can be asserted on the basis of these findings that DiOHF reduced the elevated NO values through its antioxidant effect.

Another parameter examined in our study was MDA. MDA is considered a reliable marker of lipid peroxidation [7]. Azizolazziet et al., [23] reported increased serum MDA levels in testis ischemia-reperfusion. In their study, Hanciet et al., [24] subjected rat testis to 720° torsion for one hour and detorsion for 4 hours and found a significant increase in MDA levels during I/R. Elevated tissue MDA levels were reported to result from I/R in other studies as well [21]. In this study, 720° torsion of rat testis for 4 hours increased plasma MDA values (group 3). This increase found in MDA values during ischemia and reperfusion is parallel to the results of studies reporting elevated MDA levels during I/R.

Elevated plasma and tissue MDA values in this study demonstrate that ischemia and reperfusion fully developed in the testis, as we intended at the beginning of our study. Furthermore, higher plasma MDA levels we found in the group which was subjected to both torsion and detorsion indicate that not only ischemia but also reperfusion causes significant oxidative stress. However, groups which received DiOHF together with torsion and detorsion of the same length (groups 5 and 6) were found to have lower plasma and tissue MDA values, which were, in fact, close to the values of the control group. In another study group, the animals received 4 hours of torsion and 24 hours of detorsion, but also a single-dose 30 mg/kg intraperitoneal DiOHF injection before detorsion (immediately after torsion). Previous testicular ischemia-reperfusion studies tested various flavonoids like quercetin and baicalin and suggested that these drugs could be useful in I/R injury [25]. However, as far as our literature knowledge goes, the effect of DiOHF, a synthetic flavonoid whose protective action in cardiac ischemia-reperfusion injury was explored in previous studies, on testicular ischemia-reperfusion injury has not been studied. The results of our study about MDA indicate that this parameter is elevated during and after I/R, but that administration of DiOHF, a synthetic flavonoid, may significantly reduce MDA levels, thereby attenuating the tissue damage resulting from I/R in rats.

Erythrocyte glutathione levels were also examined in this study as a marker of the antioxidant system. Glutathione is among the main intracellular antioxidant compounds that provide protection against oxidant damage and it is considered among the major elements of the organism’s antioxidant defense [26]. However, although it is accepted as an antioxidant parameter of the organism, glutathione is seen to be affected in different ways during the antioxidant defense. GSH levels were determined as a marker of tissue antioxidant activity in previous testis ischemia-reperfusion studies [21]. Likewise, Guimare et al. [27] found that GSH levels in the testis dropped as a result of 2 hours of ischemia followed by 3 hours of reperfusion. However, Unal et al., [28] showed that 2 hours of torsion increased tissue GSH levels in rats.

In the same vein, Hekimoglu et al., [15] found increased GSH values after testis ischemia-reperfusion. In our study, 4 hours of torsion significantly elevated plasma and tissue GSH levels in the experimental animals, relative to the control group, and this elevation was more marked in the tissue. An overall examination of study groups demonstrates increased tissue GSH values in torsion and detorsion groups (groups 3 and 4). This parameter was higher in the groups which were administered DiOHF together with torsion and detorsion. Increased GSH levels in ischemic groups can be attributed to the increased antioxidant response to lipid peroxidation in the tissue. Additionally, the further increase caused by DiOHF supplementation in GSH levels may be considered an indicator of the reinforced defense response to lipid peroxidation in the plasma and tissue.

4. CONCLUSIONS
Our study showed that levels of NO and MDA, indicators of oxidant damage, were elevated during ischemia-reperfusion injury in the tissue. Besides, levels of GSH, which is protective against lipid peroxidation, also increased in response to the enhanced antioxidant activity. In addition, DiOHF used in the study is seen to exercise a protective effect against lipid peroxidation in experimental ischemia-reperfusion injury by both inhibiting oxidants and strengthening the action of antioxidants. Thereafter,
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DioHF may be used after ischemia-reperfusion to prevent possible damage. And also different doses of DioHF should be try to determine which doses has affect on ischemia-reperfusion damage that we used 30 mg/kg.

5. REFERENCES


6. ACKNOWLEDGEMENTS

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