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EXPERIMENTAL STUDY

3′, 4′-dihydroxyflavonol attenuates tissue damage in unilateral testis ischemia-reperfusion in rats

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ABSTRACT
The purpose of this study was to determine the effect of 3′,4′-dihydroxyflavonol (DiOHF) on oxidative damage and antioxidant system in experimental testicular torsion-detorsion.
The study included 60 male Wistar albino rats. Study groups were formed as follows: 1. Control; 2. Sham; 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. Testis were collected for the analysis of glutathione peroxidase (GPx), nitric oxide (NO), malondialdehyde (MDA), glutathione (GSH), and xanthine oxidase (XO).
GPx in the Group 8 were higher than the values in the other groups ($p < 0.001$). Concerning NO, the groups 3, 4, and 7 were found to have higher values than other groups ($p < 0.001$). MDA levels were higher in the groups 3, 7, and 8, when compared to the levels in other groups ($p < 0.001$). When tissue GSH levels were examined, the Group 5 had the highest GSH values ($p < 0.001$).With regard to XO values, the groups 3, 4, and 7 had the highest XO values ($p < 0.001$).
The results of the study indicated that intraperitoneal DiOHF inhibited increased lipid peroxidation in testis ischemia-reperfusion injury in rats *(Tab. 5, Ref. 46)*. Text in PDF *www.elis.sk.*
KEY WORDS: testis ischemia-reperfusion, 3′,4′-dihydroxyflavonol, rat.

Introduction

Ischemia-reperfusion (IR) injury refers to the cellular damage inflicted after oxygen supply is restored to the organ that suffered hypoxia. 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 (1). Free radicals–superoxide, hydroxyl, peroxynitrite – were identified during ischemia and offer a partial explanation for the injury incurred after ischemia. However, the physiological significance of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be attributed to their ability to impact communication processes in post-hypoxic cells and the capacity of ROS to initiate programmed cell death in both apoptosis and necrosis. There is a variety of ROS sources, which produces extremely reactive species during ischemia-reperfusion (2).

Paradoxically, reperfusion of the ischemic tissue causes more severe damage than that inflicted by ischemia alone (3). Several mechanisms, including free oxygen radical (FOR) derivatives that are rapidly produced upon the entry of molecular oxygen into the cell, are involved in the damage observed during reperfusion. The cellular structures that are most susceptible to reperfusion injury are membrane lipids, proteins, nucleic acids, and deoxyribonucleic acid molecules (4). Increased intracellular Ca^{2+} ion concentration is also cytotoxic for the cell (5). Several factors have been suggested to explain the physiopathology of ischemia-reperfusion (I/R) injury. An insufficient oxygen supply to meet metabolic needs, depletion of cellular energy reserves and accumulation of toxic metabolites during ischemia cause germ cell death (6). Both reactive oxygen radicals (ROS) and reactive nitrogen derivatives like nitric oxide are significantly elevated in the reperfusion stage (5, 7).

Free oxygen radicals (FOR) that are produced during ischemia and the following reperfusion are known to be involved in the testicular damage after torsion. Causing lipid peroxidation in cell membranes, free oxygen radicals denaturalize proteins and destroy the cell (8). This destruction is ameliorated by endogenous antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase (9). Recent studies demonstrated that flavonoids had a variety of biochemical and pharmacological activities (10). They were also reported to exercise anti-inflammatory and anti-microbial effects (11).

The present study aimed to determine the effect of 3′,4′-dihydroxyflavonol (DiOHF), a synthetic flavonoid, on tissue injury in experimental testicular torsion-detorsion.

Material and methods

The present study included 60 Wistar Albino male rats supplied by the Experimental Medicine Research and Application Centre of Selcuk University and was conducted in this centre upon ap-

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proval of its ethics committee. The experimental animals weighed between 250 and 260 gr. The rats were randomized into the eight groups: two groups of six rats and six groups of eight rats. All the rats were kept in temperature- and light-controlled rooms and fed with appropriate feed and water. Surgical operations were performed after the rats were anesthetized with intraperitoneal ketamine hydrochloride (60 mg/kg) and Xylasine (rompun) (5 mg/kg).

1. General control (n = 6): The group, in which unilateral orchiectomy was performed under anaesthesia without any other procedure.
2. Sham control (n = 6): The animals in this underwent general anaesthesia (Ketamine + Rompun) and then their testicular area was surgically removed. Additionally, the animals were given an intraperitoneal DiOHF solution.
3. 720° – 4 hours torsion (n = 8): After the animals in this group underwent general anaesthesia, 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 underwent general anaesthesia, 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 underwent general anaesthesia. Then their testis were 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 underwent general anaesthesia and their right 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 undergoing general anaesthesia, 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 underwent general anaesthesia 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.

Testis tissue samples collected from the animals at the end of the procedures were stored at -80°C until the day of analysis.

Dihydroxy flavonoid injection

After being dissolved in 4 ml dimethyl sulfoxide, DiOHF (Indofine Chemical Co. USA) was added to polyethylene glycol and water to obtain a total volume of 200 ml. The solution was injected to the experimental animals at a dose of 30 mg/kg through the intraperitoneal route (12).

Tissue analyses

Glutathione peroxidase (GPx) analysis

Glutathione peroxidase was analyzed with the Cayman brand commercial kits (Catalogue no: 703102) according to colorimetric method.

Measurement of nitric oxide (NO) levels

NO levels were quantified with the Cayman brand commercial kits (Catalogue no: 780001) according to colorimetric method.

Measurement of tissue malondialdehyde (MDA) levels

Plasma MDA levels were determined according to the method suggested by Uchiyama and Mihara. The results were expressed as mg/g protein (13).

Tissue glutathione (GSH) analysis

Tissue GSH levels were measured using the Elman method. The values were presented as nmol/ gr protein (14).

Tissue xanthine oxidase analysis

In order to determine tissue xanthine oxidase values, testis samples taken from the animals were centrifuged and the samples were analyzed using xanthine oxidase activity assay kit (Bio Vision, catalogue no: K710-100) according to the colorimetric method in a spectrometer.

Statistical evaluations

Statistical evaluation of the data was conducted using the SPSS statistics software. The results were presented as the 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. The level of statistical significance was established at $p < 0.05$.

Results

Tissue glutathione peroxidase levels of the groups are presented in the Table 1. When testis tissue GPx values were examined, it was seen that this parameter was higher in the groups 5, 7, and 8, in comparison to other groups, and that the Group 8 had the highest tissue GPx levels ($p < 0.001$). The Group 7 had higher tissue GPx values than the Group 5 ($p < 0.001$).

The Table 2 shows tissue NO values in the study groups. It is seen that the groups 3, 4, and 7 had higher NO values and that NO

Tab. 1. Testes GPx Levels.

Groups	GPx (nmol/min/ml)
1–Control	7.07±1.86 d
2–Sham-Control	8.45±0.30 d
3–720°–4 hours torsion	6.47±1.15 d
4–720°–4 hours torsion+4 hours detorsion	8.23±1.10 d
5–720°–4 hours torsion+DiOHF	13.37±2.82 c
6–720°–4 hours torsion+DiOHF+4 hours detorsion	10.53±3.25 d
7–720°–4 hours torsion+24 hours detorsion	18.98±2.27 b
8–720°–4 hours torsion+DiOHF+24 hours detorsion	22.00±4.52 a

* Different letters are significant as statistic, * $p < 0.001$ (a > b > c > d)

Tab. 2. Testes NO Levels.

Groups	NO (μM)
1–Control	7.18±1.50 c
2–Sham-Control	7.62±1.66 c
3–720°–4 hours torsion	11.81±3.76 b
4–720°–4 hours torsion+4 hours detorsion	15.95±3.52 a
5–720°–4 hours torsion+DiOHF	9.71±1.52 c
6–720°–4 hours torsion+DiOHF+4 hours detorsion	9.80±2.21 c
7–720°–4 hours torsion+24 hours detorsion	14.49±3.89 a
8–720°–4 hours torsion+DiOHF+24 hours detorsion	8.97±2.46 c

* Different letters are significant as statistic, * $p < 0.001$ (a > b > c)

Tab. 3. Testes MDA Levels.

Groups	MDA (nmol/gr protein)
1–Control	7.56±1.18 d
2–Sham-Control	8.56±1.90 d
3–720°–4 hours torsion	16.83±3.00 b
4–720°–4 hours torsion+4 hours detorsion	9.50±2.40 d
5–720°–4 hours torsion+DiOHF	9.06±2.75 d
6–720°–4 hours torsion+DiOHF+4 hours detorsion	8.71±1.62 d
7–720°–4 hours torsion+24 hours detorsion	26.82±2.62 a
8–720°–4 hours torsion+DiOHF+24 hours detorsion	13.74±2.67 c

* Different letters are significant as statistic, * $p < 0.001$ (a > b > c > d)

Tab. 4. Testes GSH Levels.

Groups	GSH (mg/gr protein)
1–Control	10.72±3.02 c
2–Sham-Control	11.87±2.35 c
3–720°–4 hours torsion	17.71±6.32 b
4–720°–4 hours torsion+4 hours detorstion	18.11±4.62 b
5–720°–4 hours torsion+DiOHF	25.19±3.94 a
6–720°–4 hours torsion+DiOHF+4 hours detorsion	16.07±3.55 b
7–720°–4 hours torsion+24 hours detorsion	13.32±2.02 c
8–720°–4 hours torsion+DiOHF+24 hours detorsion	17.31±2.00 b

* Different letters are significant as statistic, * $p < 0.001$ (a > b > c)

Tab. 5. Testes XO Levels.

Groups	XO (nmol)
1–Control	1.05±0.41 c
2–Sham-Control	1.53±0.38 c
3–720°–4 hours torsion	2.37±0.62 a
4–720°–4 hours torsion+4 hours detorsion	2.67±0.94 a
5–720°o–4 hours torsion+DiOHF	1.64±0.51 c
6–720°–4 hours torsion+DiOHF+4 hours detorsion	2.14±0.24 b
7–720°–4 hours torsion+24 hours detorsion	2.97±0.30 a
8–720°–4 hours torsion+DiOHF+24 hours detorsion	2.09±0.76 b

* Different letters are significant as statistic, * $p < 0.001$ (a > b > c)

values in the groups 4 and 7 were higher than those in all other groups ($p < 0.001$).

Tissue MDA levels of the groups are shown in the Table 3. Testis tissue MDA levels were higher in the groups 3, 7, and 8 ($p < 0.001$). MDA levels in the Group 8 were lower than the levels in the Group 6, but the difference was not statistically significant.

The levels of GSH as a part of the antioxidant defense were also examined in the testis tissue. This examination revealed that the Group 5 had the highest tissue GSH levels ($p < 0.001$) (Tab. 4). Tissue GSH levels in the groups 3, 4, and 8 were higher than the levels in other groups, but lower than those in the Group 5 ($p < 0.001$).

Xanthine oxidase (XO) values of the study groups are presented in the Table 5. A comparison of XO levels showed that the groups 3, 4, and 7 had the highest tissue XO levels ($p < 0.001$). Groups and 8 were found to have higher tissue XO levels than the groups 1, 2, and 5 ($p < 0.001$).

Discussion

Ischemia and reperfusion cause damage to the vessels and organs of the ischemic area due to an inadequate vasodilation (15, 16). In this study, we primarily aimed to induce unilateral testis ischemia in the experiment groups. To do that we first gave the animals general anaesthesia. Then we rotated their right testis 720 ° clockwise and fixed it to the scrotum tissue to induce ischemia. After that, using the same ischemia period, we administered the animals DiOHF, a synthetic flavonoid, which was established to have a strong tissue protective effect in various ischemia-reperfusion studies (12, 17, 18) to examine its effect on lipid peroxidation in the testis tissue with different torsion and reperfusion durations.

As one of the most effective oxidants in the erythrocytes against stress, GPx serves important functions in phagocytic cells (19). The first parameter studied in our research was glutathione peroxidase (GPx) levels in testis tissue. Ischemia and reperfusion studies showed that GPx was affected in different ways. In the study of unilateral testicular ischemia-reperfusion injury, glutathione peroxidase levels were reported to increase with I/R (20). In the study by Guan et al., (21), on the other hand, right testis of the rats was subjected to 720° torsion for 2 hours followed by 4 hours of detorsion and, in line with our study, tissue GPx levels were found lower at the end of these procedures. Similar results were reported by Pekcetin et al (22) and Unal et al (23). In our study, GPx levels after 4 hours of testis ischemia were 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, DiOHF administration together with ischemia (I) and/or ischemia + reperfusion (I/R) in the testis tissue elevated tissue glutathione peroxidase values only in comparison to the values in the groups, which received torsion only and the control groups. Previous studies demonstrated that flavonoids were potent antioxidants and had a tissue protective effect in different tissues (24, 25, 26). In addition to their strong antioxidant effects, flavonoids were shown to be capable of preventing oxidant production (27). Differently from previous studies, our study used the synthetic flavonoid DiOHF in ischemia-reperfusion injury in the testis. The results we obtained with DiOHF in the testis tissue are supported by the results of other researchers.

In another part of our study, we examined how ischemia-reperfusion and ischemia-reperfusion + DiOHF in testis tissue affected tissue nitric oxide levels. We found that this parameter increased significantly, relative to the control group, after 4-hour ischemia. However, the group, which received 24 hours of detorsion after 4 hours of torsion (group 7), had even higher NO values. Administration of 30 mg/kg intraperitoneal DiOHF to ischemia and ischemia and reperfusion groups markedly reduced NO values, which were elevated by ischemia and reperfusion. In fact, it was suggested that nitric oxide had different effects in different I/R injuries (28). There are many studies about the changes in NO levels during testicular ischemia-reperfusion (29, 30, 31). Similar to our study, Yildiz et al (32) subjected experimental animals to 720 ° clockwise torsion for 2 hours and then to detorsion for another 2 hours before sacrificing the animals. They found that tissue NO values significantly increased in the testis, which received torsion. In another rat study, blood flow and NO secretion during testicular ischemia-reperfusion was examined using laser Doppler and nitric oxide-selective electrodes. It was shown that clamping of the testis artery markedly reduced the blood flow and that when the blood

flow was cut off, there was a significant increase in NO levels, which were then restored to normal upon removing the clamp (33, 34). Ozokutan et al (30) demonstrated that the 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. In the present study, we found significant increases in NO levels in both torsion and detorsion. Besides, NO values measured in detorsion were even higher than those measured during torsion. Increased NO levels, found due to torsion and detorsion in this study, are in parallel to the results of the studies, which reported an increase in NO due to ischemia. DiOHF used with both torsion and detorsion in this study brought about a marked decrease in plasma nitric oxide values. Thus, it can be asserted based on these findings that DiOHF reduced the elevated NO values through its antioxidant effect.

MDA is another parameter addressed in our study. MDA is considered a reliable marker of lipid peroxidation (35). In their study, Hanci et al., (36) 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 (32, 37). In this study, 720° torsion for 4 hours increased tissue MDA levels (the Group 3). Increased malondialdehyde values measured during ischemia and reperfusion are consistent with the studies, which reported that this parameter increased during I/R.

Elevated MDA levels during I and/or I/R indicate that significant oxidant damage occurs in the tissue not only during ischemia, but also during reperfusion. However, DiOHF administration together with torsion and detorsion (the groups 5 and 6) was seen to reduce tissue MDA levels significantly, almost to the level of the control group values. 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). Long-term (24-hour) detorsion following torsion, in particular, brought about a very significant increase in tissue MDA values, which were almost 3.5 times higher than the values in the control groups. However, administration of DiOHF to this group immediately after torsion and before detorsion caused a marked decrease in MDA levels. Previous testicular ischemia-reperfusion studies tested various flavonoids like quercetin and baicalin and suggested that these drugs could be useful in I/R injury (38, 39). Based on our literature knowledge, we can say that the effect of DiOHF on testicular ischemia-reperfusion injury has not been studied. The results of our study about MDA indicated that this parameter was elevated during and after I/R, but that administration of DiOHF, a synthetic flavonoid, could significantly reduce MDA levels, thereby attenuating the tissue damage resulting from I/R in rats.

Tissue levels of glutathione, an antioxidant system marker, were also investigated in this study. 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 (40). 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 (32). Likewise, Guimaraes et al (41) found that GSH levels in the testis dropped as the result of 2 hours of ischemia followed by 3 hours of reperfusion. However, Unal et a, (23) showed that 2 hours of torsion increased tissue GSH levels in rats. In the same vein, Hekimoglu et al (20) found increased GSH values after testis ischemia-reperfusion. In our study, 4 hours of torsion significantly elevated tissue GSH levels in the experimental animals, relative to the control group. An overall assessment of the study groups demonstrated an increased tissue GSH values in torsion and detorsion groups (the 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 administration in GSH levels may be considered an indicator of the reinforced defense response to lipid peroxidation in the plasma and tissue.

Another parameter explored in this study was XO in testis tissue. XO plays a critical role in different forms of ischemic and vascular diseases (42, 43). Xanthine oxidase is also a rich source of superoxide anion radicals in the blood after reperfusion. When XO part of the study was examined, it was seen that this parameter displayed a significant increase during 4-hour torsion, 4-hour torsion + reperfusion, and 4-hour torsion + 24-hour detorsion. These findings are in parallel to study results, which showed increased XO levels in ischemia (44, 45). However, administration of DiOHF both together with torsion and with torsion + detorsion markedly offset the XO increases that were seen during both ischemia and ischemia-reperfusion. The decrease found in XO levels as the result of short-term reperfusion (the Group 6) was also evident after long-term reperfusion (the Group 8). Based on our literature review, we know that DiOHF was not used in previous testis ischemia-reperfusion and lipid peroxidation studies. However, both DiOHF and some flavonoid-containing plants were used in previous ischemia-reperfusion studies of different organs (46). It was established that molecules, which had flavonoids in their structure produced a protective effect against myocardial ischemia-reperfusion injury by inhibiting XO formation (27, 46). The fact that the production of XO resulting from ischemia-reperfusion injury in the testis tissue was prevented by DiOHF administration in our study showed that the ability of flavonoids to inhibit xanthine oxidase in different tissues was evident in the testis tissue as well.

Conclusion

Our study aimed to induce ischemia-reperfusion injury in the testis tissue of rats. When the results of the study are assessed, the increase in oxidants and the suppression of the antioxidant system will attest to the full development of ischemia and reperfusion injury. In addition, DiOHF used in the study was seen to exercise a protective effect against lipid peroxidation in experimental ischemia-reperfusion injury by both inhibiting oxidants and strengthening the action of antioxidants.

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