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Research Article

Antioxidant Effects of Thymoquinone in Lower Extremity Reperfusion Injury in Rats: An Examination of SOD and MDA Levels

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Abstract

Objectives: Reperfusion injury (RI) refers to the damage caused when blood flow is restored to previously ischemic tissues. This damage is primarily due to oxidative stress. Thymoquinone (TQ), an active compound from Nigella sativa seeds, has shown antioxidant properties. This study investigates the effect of TQ on oxidative stress in a rat model of lower extremity reperfusion injury by analyzing SOD and MDA levels.

Methods: Thirty male Wistar albino rats were randomly divided into five groups: Sham (S), dimethyl sulfoxide (DMSO), ischemia-reperfusion (IR), Thymoquinone Sham (TQ-S), and IR+TQ (TQ administered intraperitoneally 1h pre-ischemia).

Results: MDA parameter increased significantly ($p < 0.001$) in the samples from the IR group compared to the S group. However, TQ treatment significantly reduced ($p < 0.05$) this increase. The mean SOD activity in the IR+TQ group exhibited the highest SOD activity compared to the S group, and the difference was significant ($p < 0.0001$).

Conclusion: The present study evaluated the effects of TQ treatment on oxidative stress in case of lower extremity reperfusion injury by measuring SOD activity and MDA levels. The findings suggest that TQ treatment may play an important role in reducing oxidative stress and preventing lipid peroxidation, thus reducing cell damage.

Keywords: Malondialdehyde, oxidative stress, superoxide dismutase, thymoquinone

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The restoration of blood flow to previously ischemic tissues or organs can lead to cell damage, known as reperfusion injury (RI). The primary causes of cell damage in this instance are believed to be oxidative stress, inflammation, and calcium overload.[1]

Malondialdehyde (MDA), a byproduct of lipid peroxidation, is a key indicator of oxidative stress, while superoxide dismutase (SOD) acts as a primary antioxidant enzyme, reducing oxidative damage by converting superoxide radicals into oxygen and hydrogen peroxide.[2,3] Both SOD and MDA levels are significantly affected by RI.

Previous studies have demonstrated that TQ, a compound derived from Nigella sativa, possesses strong antioxidants and free radical scavenging properties.[4]

This study aims to evaluate the therapeutic potential of TQ in alleviating oxidative stress during lower extremity reperfusion injury in rats, focusing on SOD and MDA levels.

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Methods

Ethical Approval

The study was conducted at the Kobay Experimental Animal Laboratory in Ankara, Türkiye, with approval from the local ethics committee. All procedures adhered to the 3Rs principle (Replacement, Reduction, Refinement) to ensure ethical treatment of the animals.

Study Design and Animals

This study was carried out on 30 male Wistar albino rats (400–450 g), which were kept under controlled conditions for 7 days in standard cages of three. The temperature was controlled within the 21–24°C range, the relative humidity was 50%, and the animals were kept under a 12-h light/dark cycle. All rats were given standard feed pellets and fresh drinking water throughout the study.

Rats were randomly divided into five groups (n=6 for each group) as outlined in Table 1.

Anesthesia and Surgical Preparation

For determining the surgical procedure, the rats were anesthetized by an intramuscular injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (50 mg/kg) before the experimental procedures. The operation was done under a heating lamp in the supine position. A transverse incision was made in the inguinal region to access the femoral artery and vein. A 26-gauge venous cannula was introduced into the main femoral vein, and intravenous heparin (100 U/kg) was administered to all rats to prevent clotting.

Ischemia-Reperfusion Procedure

For the IR groups, an atraumatic microvascular clamp was placed on the common femoral artery. Ischemia was then induced for 90 minutes, followed by reperfusion initiated following clamp removal.

Sample Collection and Analysis

Intracardiac blood samples were collected from each rat at the end of the experimental protocols under anesthesia. The samples were then subjected to analysis for relevant biomarkers (SOD and MDA) to assess oxidative stress and tissue damage.

SOD Analysis

Measurement of SOD was performed using a commercial ELISA (enzyme-linked immunosorbent assay) kit from Sunredbio brand (SunRed Biological Technology Co.Ltd, Cat No. 201-11-0169, Shanghai, China; Ref: DZE201110169, Lot: 202311). ELISA is a highly precise technique that can be used to accurately quantify the levels of SOD.[5] All reagents were diluted at room temperature according to kit protocol. SOD standards at concentrations of 64 ng/ml, 32 ng/ml, 16 ng/ml, 8 ng/ml, 4 ng/ml, and 2 ng/ml were prepared from the stock standard solution. A volume of 50 µl of each standard was pipetted into the respective wells. Since the standard solution contained a biotin-labeled antibody, no additional antibody was added to the standard wells. For the samples, 40 µl of each was added to the wells, followed by incubation with 10 µl of anti-SOD antibody. Then, 50 µl of streptavidin-HRP was added to both the standard and sample wells, and the plate was incubated at 37°C for 60 minutes. After incubation, the wells were washed five times with 350 µl of wash solution using an ELISA washer. Subsequently, 50 µl of chromogen A and chromogen B solutions were added to each well. The plate was incubated at 37°C in the dark for 10 minutes, after which the reaction was terminated by adding 50 µl of stop solution to each well. The color change (from blue to yellow) was measured at 450 nm using a microplate reader within 15 minutes.

Table 1. Experimental Groups and Protocols

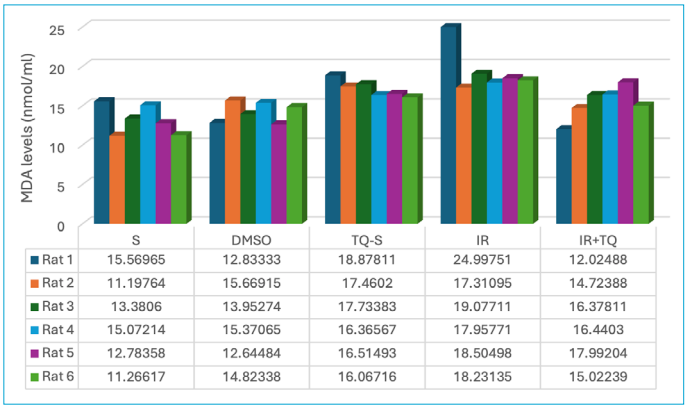
Group	Treatment	Ischemia-Reperfusion	Sample Collection
Sham Group (S, n=6)	- Inguinal incision - Femoral vein cannulation - Heparin administration	No ischemia	Intracardiac blood samples were collected 3 hours after the procedure under anesthesia.
DMSO Group (DMSO, n=6)	- Received DMSO (same volume used to dissolve thymoquinone) intraperitoneally	No ischemia	Intracardiac blood samples were collected 3 hours after the procedure under anesthesia.
Thymoquinone Sham Group (TQ-S, n=6)	- Received 20 mg/kg thymoquinone intraperitoneally	No ischemia	Intracardiac blood samples were collected 3 hours after the procedure under anesthesia.
Ischemia-Reperfusion Group (IR, n=6)	- No additional treatment	90 minutes ischemia + 90 minutes reperfusion	Intracardiac blood samples were collected after reperfusion under anesthesia.
Ischemia-Reperfusion + Thymoquinone Group (IR+TQ, n=6)	- Received 20 mg/kg thymoquinone intraperitoneally 1 hour before ischemia	90 minutes ischemia + 90 minutes reperfusion	Intracardiac blood samples were collected after reperfusion under anesthesia.

MDA Analysis

The Sunredbio brand commercial ELISA kit (SunRed Biological Technology Co. Ltd., Cat No. 201-11-0157, Shanghai, China; Ref: DZE201110157, Lot: 202311) was used to determine MDA levels.[6,7] According to the kit protocol, all reagents were brought to room temperature before being diluted. MDA standards at concentrations of 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, and 1.25 ng/ml were prepared from the stock standard solution. A volume of 50 µl of each standard was pipetted into the respective wells. Since the standard solution contained a biotin-labeled antibody, no additional antibody was added to the standard wells. For the samples, 40 µl of each was added to the wells, followed by incubation with 10 µl of anti-MDA antibody. Then, 50 µl of streptavidin-HRP was added to both the standard and sample wells, and the plate was incubated at 37°C for 60 minutes. After incubation, the wells were washed five times with 350 µl of wash solution using an ELISA washer. Subsequently, 50 µl of chromogen A and chromogen B solutions were added to each well. The plate was incubated at 37°C in the dark for 10 minutes, after which the reaction was terminated by adding 50 µl of stop solution to each well. The color change (from blue to yellow) was measured at 450 nm using a microplate reader within 15 minutes.

Statistical Analysis

The SPSS 25.0 software package (Statistical Package for the Social Sciences, Chicago, IL, USA) was used for data analysis. The normality test revealed non-parametric distributions (p=0.01), so non-parametric tests were applied. The Kruskal-Wallis test was used for statistical analyses, while the Mann-Whitney U test with Bonferroni correction, was employed to identify the group responsible for the observed differences. A significant level of 0.05 was used for decision-making. Tukey test was used to determine the statistical differences between subgroups.



	S	DMSO	TQ-S	IR	IR+TQ
Rat 1	15.56965	12.83333	18.87811	24.99751	12.02488
Rat 2	11.19764	15.66915	17.4602	17.31095	14.72388
Rat 3	13.3806	13.95274	17.73383	19.07711	16.37811
Rat 4	15.07214	15.37065	16.36567	17.95771	16.4403
Rat 5	12.78358	12.64484	16.51493	18.50498	17.99204
Rat 6	11.26617	14.82338	16.06716	18.23135	15.02239

Figure 1. MDA results.

Results

MDA Analysis

The results of the MDA analysis are presented in Figure 1. MDA levels in the IR group were significantly higher than in the Sham group (p<0.001). MDA increase was minimal in the DMSO and TQ-S groups (p<0.05), while TQ treatment significantly reduced MDA levels (p<0.05) compared to the IR group (Table 2).

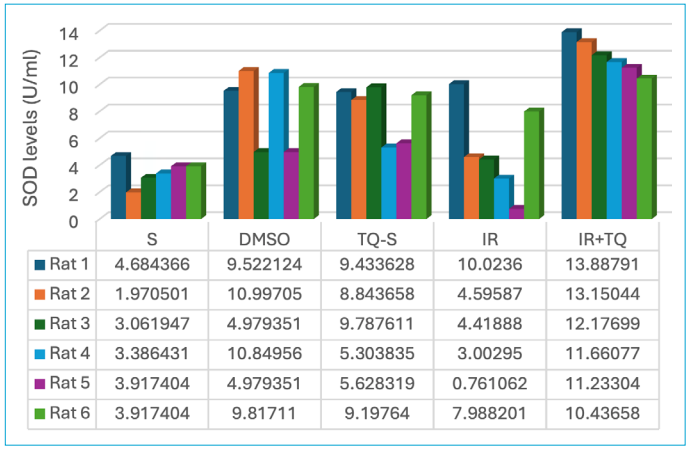
SOD Analysis

The level of SOD activity was significantly higher in the DMSO and TQ-S groups compared to the S group (p=0.0060 and p=0.0148, respectively) (see Fig. 2). The mean SOD activity in the IR+TQ group was measured as 12.09±1.266 U/ml. The IR+TQ group exhibited the highest SOD activity (p<0.0001), showing a marked reduction in oxidative stress (Table 3).

Discussion

TQ has previously been reported to reduce cell damage in IR through different pathways and mechanisms.[8-10] This is achieved by neutralizing reactive oxygen species and activating endogenous antioxidant systems such as SOD, glutathione peroxidase, and catalase. The protective effect of TQ in studies on the brain, myocardial tissue, and skeletal muscle depends on its anti-inflammatory, antioxidant, and anti-apoptotic properties. In this way, TQ positively affects clinical results by increasing cell survival. In our study, TQ's antioxidant effect was evident by the reduction in MDA and the increased activity of SOD, supporting its potential as a therapeutic agent in ischemic pathologies.

Oxidative stress is characterized by the overproduction of reactive oxygen radicals that exceed the body's antioxidant capacity. This phenomenon is recognized as



	S	DMSO	TQ-S	IR	IR+TQ
Rat 1	4.684366	9.522124	9.433628	10.0236	13.88791
Rat 2	1.970501	10.99705	8.843658	4.59587	13.15044
Rat 3	3.061947	4.979351	9.787611	4.41888	12.17699
Rat 4	3.386431	10.84956	5.303835	3.00295	11.66077
Rat 5	3.917404	4.979351	5.628319	0.761062	11.23304
Rat 6	3.917404	9.81711	9.19764	7.988201	10.43658

Figure 2. SOD results.

Table 2. MDA analysis results and statistics

Tukey's mct	Mean Diff,	95,00% CI of diff,	p
S vs. DMSO	-1.004	-4.252 to 2.244	0.8912
S vs. TQ-S	-3.958	-7.206 to -0.7105	0.0115*
S vs. IR	-6.135	-9.383 to -2.887	<0.0001*
S vs. IR+TQ	-2.219	-5.466 to 1.029	0.2921
DMSO vs. IR+TQ	-2.954	-6.202 to 0.2935	0.0874
DMSO vs. IR	-5.131	-8.379 to -1.883	0.0008*
DMSO vs. IR+TQ	-1.215	-4.462 to 2.033	0.8057
IR+TQ vs. IR	-2.177	-5.424 to 1.071	0.3098
TQ-S vs. IR+TQ	1.740	-1.508 to 4.988	0.5273
IR vs. IR+TQ	3.916	0.6685 to 7.164	0.0126*

* Statistically significant.

Table 3. SOD analysis results and statistics.

Tukey's mct	Mean Diff,	95,00% CI of diff,	Adjusted p
S vs. DMSO	-5.034	-8.878 to -1,191	0.0060*
S vs. TQ-S	-4.543	-8.386 to -0,6995	0.0148*
S vs. IR	-1.642	-5.485 to 2,201	0.7201
S vs. IR+TQ	-8.601	-12.44 to -4,758	<0.0001*
DMSO vs. TQ-S	0.4916	-3.352 to 4,335	0.9955
DMSO vs. IR	3.392	-0.4509 to 7,236	0.1024
DMSO vs. IR+TQ	-3.567	-7.410 to 0,2764	0.0782
TQ-S vs. IR	2.901	-0.9426 to 6,744	0.2064
TQ-S vs. IR+TQ	-4.059	-7.902 to -0,2152	0.0348*
IR vs. IR+TQ	-6.959	-10.80 to -3,116	0.0001*

* Statistically significant.

a primary cause of cell damage in the IR state. TQ has emerged as a subject of interest due to its bioactive nature and established antioxidant properties. This effect is achieved by the reduction of oxidative stress markers and the activation of endogenous antioxidant systems within the cell.[11-13] Furthermore, TQ has been shown to neutralize reactive oxygen molecules directly, whilst concomitantly increasing the activities of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase), thus leading to a decrease in reactive oxygen molecules.[12-15] For instance, in a study by Khiter et al., the protective effect of TQ against CCl4-induced hepatotoxicity was demonstrated by restoring SOD and catalase enzyme activities.[13] In our study, consistent with the existing literature, the antioxidant effect of TQ via SOD was demonstrated. Consequently, we hypothesize that it will reduce cell/tissue damage after reperfusion in ischemic pathologies.

Malondialdehyde is formed by reactive oxygen radicals as a result of peroxidation of polyunsaturated fatty acids in the cell membrane.[16]. MDA, a reactive aldehyde, has been shown to cause toxic effects on cells. It can be used as a biomarker to assess the level of oxidative stress.[17,18] In the present study, the level of MDA, which increased as a result of oxidative stress, was found to decrease significantly in the TQ group. This is in line with previous studies, which have observed that TQ reduces lipid peroxidation by showing an antioxidant effect.[4]

The present study evaluated the effects of TQ treatment on oxidative stress in case of lower extremity IR injury by measuring SOD activity and MDA levels. The findings suggest that TQ treatment may play an important role in reducing oxidative stress and preventing lipid peroxidation, thus reducing cell damage. The findings suggest that TQ may be beneficial in managing reperfusion injury, particularly in clinical scenarios involving ischemic damage. Nevertheless, some significant issues with TQ still require further investigation. The optimal dosage and administration timing must be established to ensure both efficacy and safety.

The present study has certain limitations. Firstly, it should be noted that the investigation was conducted solely on male Wistar albino rats. Given this, it is recommended that further studies be carried out on larger samples to evaluate the effects of sex and species differences. Secondly, only markers such as SOD and MDA were analyzed in the study. In future studies, the evaluation of inflammatory cytokines and apoptosis-related markers will help to provide a more comprehensive understanding of the protective mechanisms of TQ.

Conclusion

Thymoquinone shows promise as an effective agent in reducing oxidative stress and preventing tissue damage during reperfusion injury. Further studies are needed to optimize its therapeutic use and to explore its potential clinical applications.

Disclosures

Ethics Committee Approval: The study was conducted at the Kobay Experimental Animal Laboratory in Ankara, Türkiye, with approval from the local ethics committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authorship Contributions: Concept –T.D.; Design – T.Ö.; Supervision – T.D., T.Ö.; Materials – T.Ö.; Data collection &/or processing – T.D., T.Ö.; Analysis and/or interpretation – T.D.; Literature search – T.Ö.; Writing – T.D., T.Ö.; Critical review – T.D., T.Ö.

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