Cardioprotective effect of Thymoquinone: A constituent of *Nigella sativa* L., against myocardial ischemia/reperfusion injury and ventricular arrhythmias in anaesthetized rats

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Abstract: Reperfusion of the ischemic myocardium causes the myocardial injury and life-threatening ventricular arrhythmias in human. This study aimed to investigate the effects of thymoquinone (TQ) on myocardial ischemia/reperfusion (I/R) injury and ischemia- and reperfusion-induced ventricular arrhythmias in anaesthetized rats. Adult male Wistar albino rats were divided into two groups, each containing a control and TQ-treated subgroups. In group I, the myocardial infarct size was determined by triphenyl tetrazolium chloride staining following 2-h reperfusion preceded by 30 min of ischemia. In group II, a 6-min myocardial ischemia was followed by a 10-min reperfusion. TQ-treated subgroups were treated with TQ (10 mg/100µl/kg, i.p.) and the control subgroups were treated with the vehicle (100 µl/kg, i.p.) 20 min prior to the ischemic period. Ischemia was induced by ligating the left main coronary artery, followed by reperfusion. TQ treatment reduced the infarct size (15±4% versus 69±6%, $P<0.01$). Pretreatment with TQ decreased arrhythmia scores, as well as the incidence of ventricular tachycardia and the incidence of ventricular fibrillation during the reperfusion period (arrhythmia scores: 1.4±0.3 versus 4.4±0.3, $P<0.01$). These results suggest that TQ confers protection against myocardial I/R injury and suppresses reperfusion-induced arrhythmias.

Keywords: Thymoquinone, ischemia/reperfusion injury, ventricular arrhythmias, anaesthetized rats.

INTRODUCTION

Reperfusion provided by coronary angioplasty or thrombolysis is a standard treatment method for coronary heart patients to prevent the eventual necrosis of the ischemic myocardium, but that itself can lead to additional myocardial injury and lethal ventricular arrhythmias (Hausenloy and Yellon, 2013). The mechanisms underlying myocardial ischemia/reperfusion (I/R) injury are multifactorial. However, the generation of abundant amounts of oxygen-derived free radicals (ODFR), the inhibition of endogenous antioxidant defense mechanisms and inflammatory reactions are considered as important factors that play role in myocardial injury and the genesis of ventricular arrhythmias following ischemia and reperfusion (Frank *et al.*, 2012). Hence, protection against myocardial I/R injury can be induced by treatment including administration of antioxidant, anti-inflammatory and ODFR scavenger substances (Marczin *et al.*, 2003).

Thymoquinone (TQ; 2-isopropyl-5methyl-1,4-benzoquinone) is a pharmacologically active quinone obtained from *Nigella sativa* L. seeds, which are used in traditional (herbal) medicine as a natural remedy for the treatment and prevention of a number of diseases and conditions, including hypertension, inflammation, asthma, diarrohea, fever, headache and eczema (Ali and Blunden 2003). TQ has been reported to have strong antioxidant properties, free radical scavenging and anti-inflammatory effects (Mansour *et al.*, 2002; El Gazzar *et al.*, 2006; Woo *et al.*, 2012). It provides protection against I/R injury in various organs including cerebrum, testes and skeletal muscle (Hosseinzadeh *et al.*, 2007; Gökçe *et al.*, 2010; Hosseinzadeh *et al.*, 2012). It was found to be protective against cyclophosphamide-induced cardiotoxicity due to its antioxidant properties (Nagi *et al.*, 2011). More recently, Randhawa *et al.*, (2013) demonstrated that chronic administration of TQ significantly reduced myocardial injury induced by isoproterenol with a pronounced antioxidant activity.

It is hypothesized that with its anti-inflammatory and antioxidant characteristics (Hosseinzadeh *et al.*, 2007; Gökçe *et al.*, 2010; Hosseinzadeh *et al.*, 2012; Randawa *et al.*, 2013), TQ may protect myocardium against I/R injury and ventricular arrhythmias. The effects of TQ on myocardial I/R injury and ventricular arrhythmias have not been previously studied. Therefore, the aim of the present study is to research the possible protective action of TQ against I/R injury and ventricular arrhythmias.

METHODS

Animals

Forty-four male Wistar albino rats (10-12 month olds, weighing 330-450 g) were used in the present study. They were provided by the Experimental Animal Production and Research Centre, Başkent University/Turkey. The animals were kept in a room with a temperature of 21±2°C, 40%-65% humidity and with a 12-h light/dark

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cycle. The animals consumed tap water and standard rat pellet food ad libitum. The animals were raised and treated in experimental procedures according to the guidelines and recommendations of the World Medical Association. All of the experimental procedures in this study were discussed and approved by the Animal Research Local Ethical Committee of Bülent Ecevit University, Zonguldak (protocol no: 2011-25-28/12).

Surgical procedures

The ischemia and reperfusion protocols performed in our study were previously defined by Bozdogan *et al*. (2005). The rats were anaesthetized via an intraperitoneal (i.p.) injection of thiopental sodium (85 mg/kg) and they were put on an animal rectal temperature controller (RTC 9404-A, Commat Ltd, Ankara, Turkey) to maintain the body temperature in the range of 37±1^{o}C during the experimental period. The trachea and the left carotid artery were cannulated for artificial respiration and the measurement of arterial blood pressure (Blood pressure transducer, SS 13 L, Biopac Systems, California, USA). A standard limb lead II electrocardiogram (ECG) and arterial blood pressure were recorded and monitored throughout the experimental period (Data acquisition system MP35, Biopac System, Goleta, California, USA). The chest was opened after the fourth and fifth ribs were cut. The animal respirator was started using room air to provide artificial ventilation (60 strokes/min at a tidal volume of 1.5 ml/100 g; SAR 830, Life Science, California, USA). The hearts were exteriorised after the pericardium was incised. A 5/0 silk suture was passed around the left main coronary artery approximately 2-3 mm from its origin. The heart was then replaced and allowed to stabilize for 10 min. During this period, rats with a sustained decrease in mean arterial blood pressure (MABP) values below 70 mmHg or ventricular arrhythmias prior to the ligation were excluded. After heart rate (HR) and blood pressure stabilization, the slip loop was made using the loose ends of the previously placed silk suture. The coronary artery occlusion was induced by ligation of the coronary artery with the slip loop. The slip loop was then loosened by pulling a loose end of the loop to permit reperfusion.

ST segment elevation and increased QRS amplitude on ECG, a 20-40% reduction in arterial blood pressure compared to the pre-ischemic values and area at risk values greater than 40% were seen in all rats where a successful coronary artery occlusion was performed. A total of 14 animals were omitted on the basis of these criteria.

Experimental groups and drug treatment

Two separate experimental groups were designed to assess the effects of TQ on (i) I/R injury and ischemia-induced arrhythmias (group I) and (ii) reperfusion-induced arrhythmias (group II).

Group I: 30 min of ischeamia followed by 2h of reperfusion

This portion of the experiment involved 20 animals that were randomly divided into two subgroups: a control group and a TQ-treated group. The duration of ischemia and reperfusion was 30 min and 2 h, respectively, in the animals belonging to group I. Because reperfusion arrhythmias can be elicited by different mechanisms from those of ischemia (Curtis *et al*., 1993), the model of a 30 min left coronary artery occlusion followed by 2h reperfusion is not appropriate for evaluation of reperfusion-induced arrhythmias (Lee *et al*., 2002). Therefore, the effects of TQ on reperfusion-induced arrhythmias were examined in group II.

Group II: 6 min of ischeamia followed by 10 min of reperfusion

This portion of the experiment involved 24 animals that were randomly divided into two subgroups: a control group and a TQ-treated group. Ischemia was applied for 6 min and reperfusion for another 10 min in the animals in group II. This model was used to evaluate reperfusion-induced arrhythmias because the reperfusion following 6 min of ischemia induced various ventricular arrhythmias, ranging from other types of arrhythmias (ventricular premature contraction [VPC]) including bigeminy, salvos and single extra systoles to ventricular fibrillation (VF), and caused extended arrhythmic attacks in our previous studies (Gonca and Bozdoğan, 2010; Gonca, 2013). Pure powder TQ was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The TQ dose and the administration route used in the present study are based on previous studies (Hosseinzadeh *et al*., 2007; Gökçe *et al*., 2010). TQ was dissolved in dimethyl sulfoxide (DMSO), followed by the addition of 0.9% saline solution (1:1) on a daily basis and i.p. administered at a dose of 10 mg/100µl/kg 20 min prior to ligation in the drug-treated groups. In the control groups, DMSO and saline (1:1) at a volume of 100 µl/kg was administered via the same route and timing.

Measurement of the area at risk

The area at risk was measured in both groups. After the reperfusion period, the heart was removed and cannulated through the aorta before being subjected to retrograde perfusion with 10 ml of saline solution at 37°C. The heart was then perfused with 2 ml of 96% ethanol to specify the area- at risk, followed by reocclusion of the left coronary artery. The non-ischemic areas of the heart were thoroughly perfused with ethanol and appeared white in colour. The areas that were not perfused with ethanol were defined as the area at risk and remained red in colour (original tissue colour). The atrias were removed and the ventricles stored at -20°C for 15 minutes, before being sliced into five pieces of approximately 2 mm thickness and placed between two glass plates. The area at risk and total area were traced on a sheet (fig. 1).

Measurement of infarct size

The size of the infarcted area was measured in group I. In this group, the risk areas of the slices were separated from those that were well perfused with ethanol. Those slices that only consisted of the areas at risk were then stained with 1% triphenyl tetrazolium chloride (TTC) (Sigma Aldrich) and fixed in 10% formaldehyde solution overnight. The slices were placed between two glass plates. TTC stained living myocardial tissue in deep red colour, while necrotic tissue was not stained with TTC and appeared as tan in colour. The area at risk and the infarct areas were traced on acetate sheets from these plates (fig. 2).

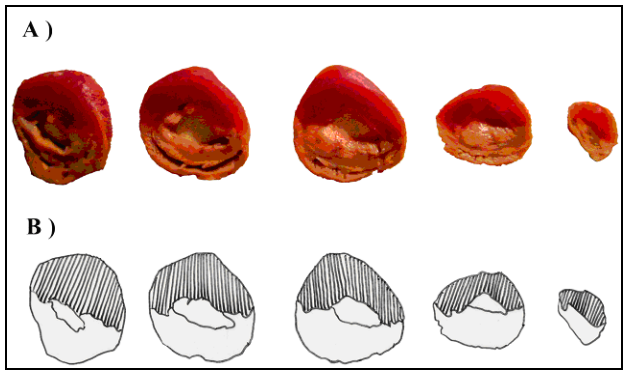

Fig. 1: Example of the measured area at risk. A) The non-ischemic area appears white in color because of the presence of ethanol, the ischemic area (area at risk) seems red in color. B) Total left ventricular area and the area at risk were traced on an acetate sheet. The shaded areas are the areas at risk.

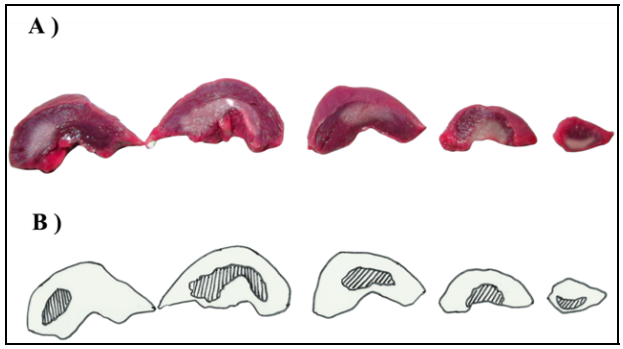

Fig. 2: Example of the measured cardiac necrosis (infarct size). A) The infracted area appears pale and white in color. B) The area at risk and infarcted areas were traced on an acetate sheet. The shaded areas are infracted regions.

Data analyses

ECG and blood pressure recordings were analyzed to determine the MABP and HR parameters at regular intervals throughout the ischemia and reperfusion periods using a data acquisition system (MP 35, Biopac system, Goleta, California, USA).

In accordance with the Lambeth Conventions (Curtis *et al.*, 2013), arrhythmias were identified during the I/R periods as VF, ventricular tachycardia (VT) and other types of arrhythmias (VPC) including bigeminy, salvos and single extra systoles (fig. 3). A grade was given to each animal as an index of the severity of arrhythmias (arrhythmia score) according to a scale as follows: 0 - no arrhythmia; 1- in the absence of VF, the duration of VT and/or VPC is shorter than 10 s or equal to 10 s; 2- in the absence of VF, the duration of VT and/or VPC is between 11 and 30 s; 3- in the absence of VF, the duration of VT and/or VPC is between 31 and 90 s; 4- the duration of reversible VF is shorter than 10 s or equal to 10 s and/or the duration of VT and/or VPC is between 91 and 180 s; 5- the duration of VF is longer than 10 s and/or the duration of VT and/ or VPC is longer than 180 s ; and 6- irreversible VF (Lepran *et al.*, 1983). The incidence of arrhythmia types and mortality, the arrhythmia scores, the durations of arrhythmic attacks and arrhythmic periods, which are the time intervals between the onset and the end of the arrhythmias, were determined in both the groups.

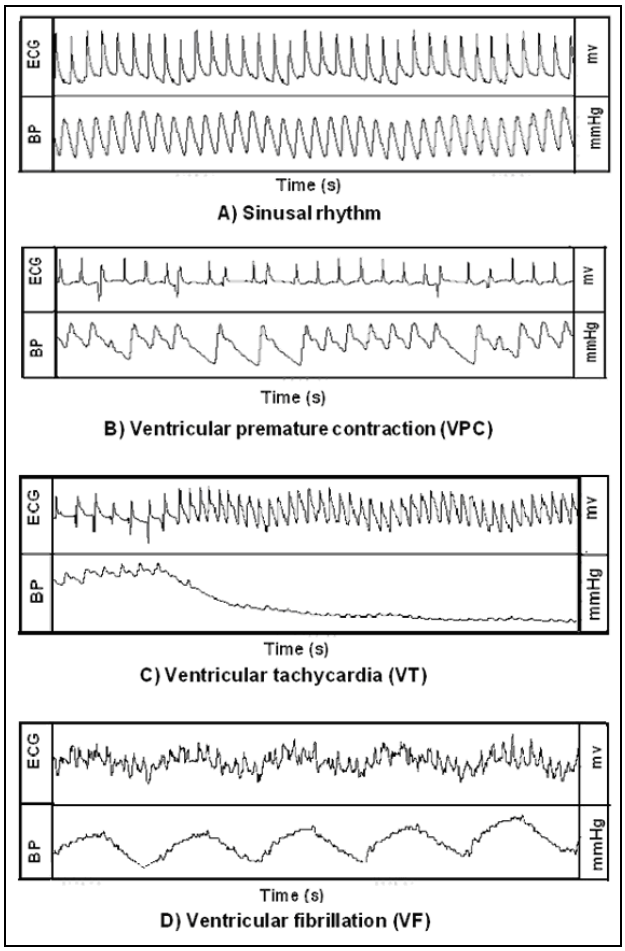

Fig. 3: Original electrocardiogram (ECG) tracings and blood pressure (BP), (recorded with speed 80 mm/s): (A) Sinusal rhythm, (B) Ventricular premature contraction (VPC), (C) Ventricular tachycardia (VT), (D) Ventricular fibrillation (VF).

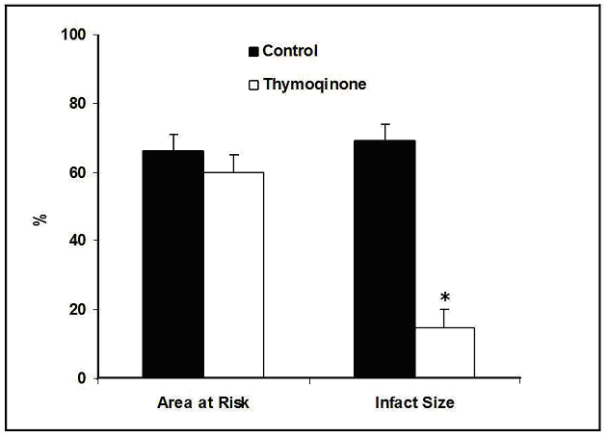

Fig. 4: Effect of TQ on area at risk and infarct size. Area at risk was expressed as a percentage of total left ventricular area and the infarct size was expressed as a percentage of area at risk. Both sets of data are expressed as the mean±SE (n=6). *P<0.001: Compared to control.

The sheets for the areas at risk and infarcted areas were scanned and transferred to a computer to measure the left ventricular area, the area at risk and the infarct size using an image processing program (ImageJ Software, National Institute of Health (NIH), Maryland, USA). The area at risk and the infarct size were determined as a percentage of the total left ventricular area and as a percentage of the area at risk, respectively.

STATISTICAL ANALYSES

Data were analysed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). The drug-treated subgroups were compared with the corresponding controls in each group. Fischer's exact test was performed for statistical analyses of mortality and the incidence of arrhythmias. All other data was expressed as means with ± standard error of the mean. Mann-Whitney U test was used to analyse the arrhythmia scores. All other parameters were analysed with Student's two-tailed t-test. Pre- and post-occlusion MABP/HR values were also compared using Student's two-tailed t-test. Changes with P values of less than 0.05 were considered to be significant.

RESULTS

ST segment elevation and the QRS voltage increment were observed following ligation. ECG changes disappeared following reperfusion in all animals. The weights of the animals did not differ between the subgroups in both the groups.

The effects of thymoquinone on hemodynamic parameters

The effects of TQ on hemodynamic data, including HR and MABP, during a 30-min ischemia period, followed by a 2-h reperfusion, and a 6-min ischemia period, followed by a 10-min reperfusion, are summarized in tables 1 and 2, respectively. The characteristic fall in MABP was observed following ligation in all the subgroups (P<0.05) (tables 1 and 2). The MABP recovered and approached the pre-ischemic values after the 10^{th} and 30^{th} min of ligation in group I (table 1) and after the 5^{th} min of ligation following reperfusion in group II (table 2). TQ treatment did not significantly affect HR and MABP at all time points in both the groups.

The effect of thymoquinone on ischemia-induced arrhythmias

Myocardial ischemia resulted in ventricular arrhythmias, which commenced 4-15 min after occlusion and occurred as VT and VPC. The number of animals, which were applied successful operations, was 7 and 6 in the control and TQ-treated subgroups, respectively. One animal in the control subgroup died as a result of irreversible ventricular fibrillation during the ischemia period. TQ treatment did not affect the incidence of arrhythmias, the duration of any type of arrhythmias or the total length of arrhythmias compared to control during 30 min of ischemia (total length of arrhythmias: 38±7 s versus 34±14 s, not significant). Myocardial reperfusion resulted in ventricular arrhythmias occurred as VPC. TQ treatment did not affect the incidence and duration of arrhythmias when the animals were subjected to a 2-h reperfusion period followed 30-min of ischemia (VPC: 8±8 s versus 12±9 s, not significant).

Table 1: The summary of heart Rate (HR) and mean arterial blood pressure (MABP) values during ischemia and reperfusion in group I (Results: Mean ± SE).

Time	Control	Thymoqinone
MABP (mm Hg)		
0 (Basal)	103±5	96±3
1 (Lig 1 min)	76± 6*	68± 3*
10 (Lig 10 min)	80±12	88±6
30 (Lig 30 min)	86±13	79±11
35 (Rep 5 min)	65±12	73±8
90 (Rep 60 min)	72±15	78±9
150 (Rep 120 min)	67±12	62±10
HR		
0 (Basal)	393±9	366±22
1 (Lig 1 min)	409± 9	368 ±37
10 (Lig 10 min)	353±39	337±34
30 (Lig 30 min)	352±43	333±18
35 (Rep 5 min)	299±50	325±33
90 (Rep 60 min)	345±40	329±26
150 (Rep 120 min)	350±40	320±25

**P<0.05*: Compared to pre-ischemic values. N: 6-6.

Effects of thymoquinone on infarct size

Fig. 4 illustrates the effects of TQ on both area at risk (percentage of LV) and infarct size (percentage of AAR). The area at risk did not differ between the subgroups. TQ treatment significantly decreased infarct size compared to control (15±4% versus 69±6%, P<0.01) (fig. 4).

Table 2: The summary of heart rate (HR) and mean arterial blood pressure (MABP) values during ischemia and reperfusion periods in group II (Results: Mean ± SE).

Time (min)	Control	Thymoqinone
MABP (mm Hg)		
0 (Basal)	88±5	93±5
1 (Lig 1 min)	60±7*	71±5*
5 (Lig 5 min)	78±16	95±5
11 (Rep 5 min)	86±14	95±3
15 (Rep 9 min)	81±16	92±5
HR		
0 (Basal)	370±19	356±10
1 (Lig 1 min)	367±16	360±12
5 (Lig 5 min)	372±20	365±16
11 (Rep 5 min)	352±31	357±11
15 (Rep 9 min)	342±41	353±10

***P<0.05: Compared to pre-ischemic values. N: 6-7.

Effects of thymoquinone on reperfusion-induced arrhythmias

The area at risk values did not differ significantly between the subgroups (table 4). Myocardial ischemia resulted in ventricular arrhythmias, which commenced 4-6 min after occlusion and occurred as VPC. TQ treatment did not affect the incidence and duration of arrhythmias during a 6-min ischemic period (VPC: 3±1 s versus 4±2 s, not significant). Myocardial reperfusion resulted in severe ventricular arrhythmias, which started between 0 and 30 s after reperfusion. TQ treatment decreased the incidence of VF and VT and arrhythmia scores (arrhythmia scores: 1.4±0.3 versus 4.2±0.5, P<0.01) (table 3) as also the lengths of VT, VPC, their total lengths and periods (total lengths of arrhythmias: 13±6 s versus 104±40 s, P<0.01) (table 4).

DISCUSSION

The results of the present study demonstrate that TQ treatment at a sublethal dose (Al-Ali *et al*., 2008) (10 mg/kg 20 min prior to ischemia) conferred protection against myocardial I/R injury and reperfusion-induced lethal ventricular arrhythmias in anaesthetized rats. The cardioprotective effects of TQ against I/R injury and ventricular arrhythmias have not been previously reported, and hence a comparison is not possible with the results of the present study.

The production of ODFR following the reperfusion of the ischemic myocardium, in combination with decreases in antioxidant activity, causes tissue injury and the generation of lethal ventricular arrhythmias (Moens *et al*., 2005). Activated neutrophils in the reperfused myocardium are important sources of the ODFR. Therefore, ODFR scavengers, antioxidants and neutrophil inhibitors are important pharmacological tools that can be used to treat myocardial I/R injury and suppress ischemia and I/R-induced arrhythmias (Wang *et al*., 2002).

TQ treatment has been shown to decrease I/R injury in various tissues, including the cerebrum, testes and skeletal muscle (Hosseinzadeh *et al*, 2007; Gökçe *et al.*, 2010; Hosseinzadeh *et al.*, 2012). TQ was also found to display protective effects against cyclophosphamide- and doxorubicin-induced cardiotoxicity (Nagi and Masour, 2000; Nagi *et al.*, 2011). In these studies, the protective effects of TQ were suggested to be dependent upon its ability to decrease oxidative stress and preserve the activity of antioxidant enzymes.

In the present study, the significantly reduced infarct area observed in TQ-treated rats following I/R is in line with the results of more recent study (Randhawa *et al.,* 2013). In this study, the chronic administration of TQ reversed the changes in histopathology of rat hearts with a pronounced antioxidant activity in the model of isoproterenol-induced myocardial infarction. In a recent study, TQ was administered i.p. at a single dose of 10 mg/kg (as in the present study) and was reported to suppress oxidative stress and decrease testicular I/R injury in mice (Gökçe *et al*., 2010). In the present study, TQ may have also demonstrated antioxidant and free radical scavenging effects in I/R arrhythmia model in anaesthetized rats, and the cardioprotective effects of TQ against I/R injury and reperfusion-induced arrhythmias may be dependent on its these actions. TQ has also been shown to have anti-inflammatory effects (El Gazzar *et al.*, 2006; Woo *et al.*, 2012). Because the activated neutrophils in the reperfused myocardium are important sources of ODFR (Pasnik and Zeman, 2009), anti-inflammatory substances may have protective effects against I/R injury and reperfusion-induced arrhythmias (Gonca, 2013). Therefore, in the present study, the cardioprotective effects of TQ may have been partially mediated via its anti-inflammatory effects.

In the present study, the ineffectiveness of TQ against ischemia-induced arrhythmias may explain the role of ODFR in the generation of ischemia- and reperfusion-induced arrhythmias to different extents. Free radical formation occurs under ischemic conditions despite reduced availability of oxygen. However, far greater production of the superoxide radical would occur on reperfusion if oxygen were returned at much higher concentrations (Rao *et al*., 1983). Although the production of ODFR is responsible for the generation of ventricular arrhythmias during both the periods, it is more effective in generating reperfusion-induced arrhythmias compared to

Table 3: The effects of Thymoqinone on arrhythmia score and the incidence of arrhythmias during 6 min of reperfusion

Group	N	Mortality n/%	Arrhythmia incidence (*n* /%)			Arrhtythmia score
			VF	VT	VPC	
Control	10	4/40	5/50	10/100	9/90	4.2±0.5
Thymoqinone	7	0/0	0/0*	3/43*	7/100	1.4±0.3**
P-value			0.0441	0.0147		0.0011

VF: Ventricular fibrillation; VT: Ventricular tachycardia; VPC: Extrasystoles, salvos, bigeminy N: The number of animals just before the reperfusion n: The number of dead animals after 6 min of reperfusion. *n*: The number of animals experienced arrhythmias *$P<0.05$; **$P<0.01$: Compared to control.

Table 4: The Effects of Thymoqinone on the duration of arrhythmias during 6 min of reperfusion

Group	N	Area at Risk (% of Total)	Arrhythmic Period (s)	Length of Arrhythmic Attacks (s)			
				VF	VT	VPC	Total
Control	6	53±2	332±82	3±2	55±31	46±13	104±40
Thymoqinone	7	55±3	102±31*	0±0	1±1**	12±5*	13±6**
P-value			0.0178		0.0034	0.0465	0.0081

N: The number of survived animals after 6 min reperfusion. VF:Ventricular fibrillation; VT: Ventricular tachycardia; VPC: extrasystoles, salvos, bigeminy; Total: The total length of VF, VT and VPC. Values represent mean ± SE, *$P<0.05$; **$P<0.01$: Compared to control.

ischemia-induced arrhythmias (Sedlis., 1992). Therefore, it is plausible to expect that antioxidant therapy would be more effective in decreasing reperfusion-induced arrhythmias than ischemia-induced arrhythmias.

CONCLUSIONS

The results of the current study reveal for the first time that TQ pretreatment decreases I/R injury and suppresses reperfusion-induced ventricular arrhythmias. Further studies are needed to clarify the mechanisms underlying its antiarrhythmic and tissue protective effects. However, it can be speculated that the cardioprotective effects of TQ may be dependent upon its antioxidant, ODFR scavenging, or anti-inflammatory effects, or all of the three combined.

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