The effect of thymoquinone, an active component of *Nigella sativa*, on isoproterenol induced myocardial injury

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Abstract: Myocardial injury constitutes a major cause of morbidity and mortality in humans. Present study aimed to investigate protective role of thymoquinone, which is an active principle of *Nigella sativa* (*N. sativa*) seed (Commonly called as black seed), in isoproterenol induced myocardial injury, a classical example of excess catecholamines related coronary insufficiency and 'stress cardiomyopathy'. Thymoquinone, in olive oil, was administered orally (12.5, 25 and 50mg/kg) to three groups of Wistar albino rats for 7 days, while two control groups were given plain olive oil. Thereafter, thymoquinone receiving groups and one control group were injected, subcutaneously, with isoproterenol (125mg/kg) for 2 days. Myocardial injury was assessed by biochemical markers (plasma LDH, TBARS, GR & SOD and myocardial GSH/GSSG ratio) and cardiac histopathology. Plasma LDH, TBARS and GR increased in control groups receiving isoproterenol, while there was a dose related decrease in these markers in thymoquinone treated groups, down to levels in controls given olive oil only. Decrease in plasma SOD and myocardial GSH/GSSG ratio and histological changes produced with isoproternol were also reversed in thymoquinone treated rats. Results of our study revealed that thymoquinone protects the heart from injury induced by isoproterenol.

Keywords: Thymoquinone, *Nigella sativa*, isoproterenol, oxidative stress, cardioprotection.

INTRODUCTION

Natural catecholamines in the body, like adrenaline and noradrenaline, have an important role in the regulation of contractility of the myocardium as well as its metabolism. However, their excess has been shown to cause biochemical changes and damage in the myocardial cells of the heart (Raab, 1960), manifested as angina and myocardial infarction. Myocardial damage induced by catecholamines is an excellent example of 'stress cardiomyopathy' (Selye, 1970). The term 'stress cardiomyopathy' is also used to explain sudden death due to stressful situations in life (Cibllis and Hirstat, 1980). Athough the process of myocardial necrosis induced by catecholamines is multifactorial, oxidative stress seems to play a major role (Dhalla *et al.*, 1978; Arti *et al.*, 2007).

Adaptive changes in the myocardial cells which provide protection to the heart have drawn a great attention of scientists. One of these important changes is an increase in the antioxidants (like glutathion peroxidase, superoxide desmutase and catalase) and other proteins (like heat shock proteins) in the myocardium (Das *et al.*, 1995; Mala and Takeuchi, 2008). Increases in the antioxidants are perhaps more directly involved in the cytoprotective effects, particularly in the stress-induced myocardial injury (Bensard *et al.*, 1990).

Nutritional and herbal medicine products have been demonstrated to play an important role in reducing the incidence of cardiovascular diseases, particularly caused by oxidative stress (Hornstra *et al*., 1998). Prolonged administration of raw garlic was shown to increase endogenous antioxidants as well as reduce lipid peroxidetion in rat heart (Banerjee *et al*., 2002). Freshly crushed garlic was found to be superior to processed garlic in terms of cardio-protection in ischemic/reperfused rat heart model. The ability of cardioprotection in garlic is attributed to S-allylcysteine, which is converted to allicin and finally broken down to hydrogen sulfide (Mukherjee *et al*, 2009).

The *N. sativa* seed, known as 'Black Seed' in English, 'Al-habba Al-sauda' in Arabic and 'Kalvanji' in Urdu and Hindi languages, is frequently used in many parts of the world, particularly in the Middle-East and Far-East countires, for the prevention and treatment of a large number of diseases (El-Kadi and Kandil, 1986). The *N. sativa* seed and its active constituents possess many pharmacological properties, including antioxidant, antiinflammatory, analgesic, antipyretic, antiasthmatic, antihypertensive, antimicrobial and antineoplastic (reviewed in Randhawa and Alghamdy, 2002; Padye *et al*, 2008; Randhawa and Alghamdy, 2011). Most of the biological effects of *N. sativa* seed are shown to be due to its major active principle, thymoquinone. The results of acute and subchronic toxicity studies of thymoquinone in the laboratory animals report it to be quite safe, especialy when given orally (Badary *et al*., 1998; Mansour *et al*., 2001; Al-Ali *et al*., 2008).

Thymoquinone, because of antioxidant properties, was shown to ameliorate acute endotoxemia-induced liver

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dysfunction and cyclophosphamide-induced cardiotoxicity in rats (Helal, 2010; Nagi *et al*, 2011). Thymoquinone alone and along with proanthocyanidin also attenuated streptozotocin induced diabetic nephropathy in rats (Sayed, 2012). Since, isoproterenol induced myocardial injury is a classical example of excess catecholamines related coronary insufficiency and stress cardiomyopathy', therefore, the present study was designed to investigate whether thymoquinone could protect the heart from damage induced by isoproterenol in rats. The research project (No: 90064) was sponsored by the Deanship of Research, University of Dammam, Dammam, Saudi Arabia and approved by the Ethical Committee of the said university.

MATERIALS AND METHODS

Animals

Male Wistar albino rats bred in the animal house of the University of Dammam, and weighing between 125 and 275g were used in the study. They were kept under standard laboratory conditions at a room temperature of 25 ± 2°C, relative humidity of 50 ± 15% and normal light period (12 hour light and 12 hour dark). Rats could freely take pellet diet and water.

Thymoquinone and olive oil

Thymoquinone was obtained from SIGMA (Aldrich), USA and olive oil from the local market (originaly a product of Italy and packed for Sasso via Benvenuto Cellini, Tavarnelle Val di Pesa, Italy). Stock solutions of thymoquinone in olive oil were prepared in concentrations of 250mg, 125/mg and 62.5 mg/ml; and 1ml/200 g rat of each was administered by oral gavage daily for 7 days to achieve the doses of thymoquinone 50, 25 and 12.5mg/kg/day, respectively. These doses were approximately 1/100 to 1/20 of LD50 values for thymoquinone in rats reported in the literature (Mansour *et al*., 2001; Al-Ali *et al*., 2008).

Isoproterenol

Isoproterenol was also obtained from SIGMA (Aldrich), USA. Fresh solution of 625mg/ml was prepared in sterile distilled water daily and its 1ml given subcutaneously (SC) per 200g rat once daily for two days to achieve a dose of 125mg/kg/day.

Experimental groups

A. Isoproterenol 125 mg/kg/day, SC two doses at 24 hour interval (active controls)

B. Thymoquinone-I + Isoproterenol (pretreated orally with 12.5mg/kg/day of thymoquinone in olive oil for 7 days and then subjected to isoproterenol 125 mg/kg/day in 1ml, SC two doses at 24 hour interval)

C. Thymoquinone-II + Isoproternol (pretreated orally with 25 mg/kg/day of thymoquinone in olive oil for 7 days and then subjected to isoproterenol 125 mg/kg/day in 1ml, SC two doses at 24 hour interval)

D. Thymoquinone-III + Isoprotereno (pretreated orally with 50 mg/kg/day of thymoquinone in olive oil for 7 days and then subjected to isoproterenol 125 mg/kg/day in 1ml, SC two doses at 24 hour interval)

E. Olive oil (1ml) alone orally for 7 days, No TQ or ISO, (passive controls)

Experimental procedure

The study was conducted on randomly distributed five groups of rats (mentioned above), each comprising of six rats. Three groups received TQ in olive oil orally at 10:00 am for 7 days in three different doses, 12.5, 25 and 50mg/kg. Two groups (a passive and an active control) were given an equivalent volume of olive oil daily for 7 days. At the end of 7-day treatment period, all rats were injected, subcutaneously, isoproterenol 125 mg/kg dissolved in distilled water for two days, except the passive controls, and sacrificed 24 hour after the last injection (Rona, 1985). Hearts of four rats from each group were removed, immediately frozen with liquid nitrogen and stored at -80°C for biochemical tests (myocardial reduced and oxidized glutathion, GSH/GSSG, ratio). While hearts of another two rats from each group were stored in 10% buffered formalin for microscopic examination. In four rats from each group, before removing the heart, 2 ml of blood was taken from the left ventricle, collected in a heparinized vials and centrifuged at 3000×g for 10 minutes. The plasma was separated and stored at -80°C for the measurement of plasma Lactate Dehydrogenase (LDH), Thiobarbituric Acid Reactive Substances (TBARS), Glutathione Reductase (GR) and Superoxide Desmutase (SOD).

Biochemical parameters

Plasma LDH

LDH was measured by the COBAS INTEGRA Lactate Dehydrogenase acc. IFCC ver.2 reagent system (Cat. No. 03004732 122) using cobas integra 400 plus analyzer. In this method the rate of NADH formation, which is directly proportional to the catalytic activity of LDH, is estimated by an increase in the absorbance at 340 nm.

Plasma TBARS

Plasma TBARS, thiobarbituric acid reactive substances, were measured by Cayman's TBARS Assay kit. The method is based on colorimetric measurement of malondialdehyde and thiobarbituric acid (MDA-TBA) adduct at 530-540 nm, formed by the interaction of MDA and TBA at the temperature of 90-100°C in acidic medium.

Plasma GR

Plasma GR was determined by the Calbiochem Glutathione Reductase Assay Kit (Cat. No. 359963) and is based on the oxidation of NADPH to NADP, causing a

Table 1: Results of various biochemical parameters in rats administered different doses of thymoquinone and controls (n=4). Demonstrates reversal of isoproterenol induced increase in LDH, TBARS and GR activity as well as reversal of decreased SOD activity and GSH/GSSG ratio by different doses of Thymoquinone (TQ)

Biochemical Parameter	Controls (Passive)	Controls (Isoproterenol)	TQ-Dose I (12.5mg/kg)	TQ-Dose II (25mg/kg)	TQ-Dose III (50mg/kg)
LDH (U/L)	689.3± 103.1	3521±43 p<0.001 vs Passive control	2676±178 p<0.05 vs. Isoproterenol	1484±291 p<0.01 vs. Isoproterenol	935±123 p<0.001 vs. Isoproterenol
TBARS activity (µmole/L)	3.4±0.6	5.9 ± 0.7 p<0.003 vs. Passive control	5.34±0.69 p<1.0 vs. vs. Isoproterenol	4.5±0.5 p<0.594 vs. Isoproterenol	3.7 ± 0.3 p<0.01 vs. Isoproterenol
GR activity (nmol/min//ml)	20.1±1.3	79.3±28.7p<0.007 Vs. Passive control	31.5±13.7p<0.036 vs. Isoproterenol	24.1±5.4p<0.012 vs. Isoproterenol	17.4±3.4p<0.005 vs. Isoproterenol
SOD activity (U/ml)	0.153±.018	0.048±0.003 p<0.001 vs Passive control	0.056 ± 0.005 p<0.1 vs. Isoprotereno	0.072 ± 0.009 p<0.07 vs. Isoproterenol	0.163 ± 0.019 p<0.001 vs. Isoproterenol
GSH/GSSG ratio	33.6±2.6	9.9±0.3 p<0.001 Vs. Passive control	12.8±0.5 p<1.0 vs. Isoproterenol	16.9±2.7 p<0.05 vs. Isoproterenol	32.2±2.8p<0.001 vs. Isoproterenol

decrease in absorbance at 340 nm, which is proportional to the activity of GR.

Plasma SOD

The plasma SOD, important for the prevention of stress related conditions, was calculated using Calbiochem Superoxide Desmutase Assay Kit II (Cat. No. 574601). In this method superoxide radicals generated by xanthine oxidase and hypoxanthine are estimated by the amount of tetrazolium salt utilized. After processing the samples and standards by the prescribed procedure the absorbance is measures at 450 nm on a plate reader.

Myocardial GSH/GSSG ratio

Calbiochem GSH/GSSG ratio Assay Kit (Cat. No. 371575) was used to estimate the GSH/GSSG ratio, which decreases due to oxidative stress in myocardial cells and is used to measure the antioxidant effect of intervening compounds. A small piece of heart from the frozen samples was weighed, immediately mixed in ice cold phosphate buffer (to give 10mg/ml), homogenised and used for the estimation of GSH and GSSG by the prescribed procedure and their ratio determined by the following formula:

$$\text{GSH/GSSG Ratio} = \frac{\text{GSH } 2\text{GSSG}}{\text{GSSG}}$$

Histopathology

Hearts of rats fixed in 10% formalin were processed by routine method and fixed in paraffin. Paraffin sections were made on glass slides and stained with hematoxylin and eosin. Finally, the slides were observed under a microscope (X40).

STATISTICAL ANALYSIS

Mean ± S.D. was calculated for all biochemical tests in different groups of rats. These values were compared by one way ANOVA as well as by Bonferroni test. The P values were determined to estimate the significance of difference between different test groups versus active and passive controls.

RESULTS

Biochemical Parameters

The results of various biochemical parameters in rats administered different doses of thymoquinone and controls are given in table 1.

Plasma LDH

In control rats given isoproterenol the LDH level increased 4-5 times the passive controls. Whereas, in rats administered thymoquinone 12.5, 25 and 50mg/kg for 7 days, LDH levels decreased with an increase in the dose and 50mg/Kg dose reduced LDH levels to near normal.

Plasma TBARS

In control rats given isoproterenol the TBARS activity increased almost 2 times the controls without any drug. Whereas, in rats administered thymoquinone 12.5, 25 and 50mg/kg for 7 days, the TBARS activity decreased with an increase in dose and with 50mg/kg the TBARS activity came down to near normal, reflecting an antioxidant effect of thymoquinone by preventing lipid peroxidation in the heart.

Plasma GR

In control rats given isoproterenol the GR activity increased almost 4 times the controls without drug. Whereas, in rats administered thymoquinone 12.5, 25 and 50mg/kg for 7 days, the GR activity decreased in a dose related manner, showing a protective effect of thymoquinone.

Plasma SOD

In control rats given isoproterenol the SOD activity

decreased almost 4 times the controls without drug. Whereas, in rats administered thymoquinone 12.5, 25 and 50mg/kg for 7 days, the SOD activity increased in a dose related manner and returned to almost normal levels, showing a protective effect of thymoquinone.

Myocardial GSH/GSSG Ratio

The myocardial GSH/GSSG ratio decreased in controls receiving isoproterenol due to oxidative stress. Whereas, the ratio gradually returned to near normal levels with corresponding increases in the dose of thymoquinone; demonstrating its antioxidant effect.

Histopathology

Patchy areas of mild necrosis with the infiltration of polymorphonuclear cells was observed in the hearts of control rats receiving isoproterenol under the light microscope (40X). No significant changes were observed in rats pretreated with different doses of thymoquinone and the histological appearance of their hearts was similar to those of passive controls.

DISCUSSION

The protective effect of *N. sativa* seed extracts and thymoquinone against noxious stimuli has been reported by a number of investigators in different tissues. For example, thymoquinone protected isolated rat hepatocytes against ter-butyl-hydro-peroxide-induced toxicity (Daba and Abdel-Rehman, 1998) and ameliorated cardiotoxic effect of doxorubicin without decreasing its antitumor activity in intact rats (Al-Shabanah *et al*, 1998). Similarly, thymoquinone attenuated gentamicin-induced nephrotoxicity (Sayed-Ahmed and Nagi, 2007) and *N. sativa* oil protected cyclosporine-A caused myocardial injury in rats (Ebru *et al*, 2008**).** Moreover, *N. sativa* oil and thymoquinone were demonstrated to prevent liver from toxicity produced by cyclophosphamide (Alenzi *et al*, 2010)**.**

The present study demonstrates the protective effect of thymoquinone against isoproterenol produced changes in biochemical parameters and histopathology. In the control groups receiving isoproterenol the plasma levels of LDH, TBARS and GR were increased 2 to 5 times the passive controls. Pretreatment with thymoquinone 12.5, 25 and 50mg/kg for 7 days, significantly decreased these biochemical markers in a dose related manner, down to their levels in the passive controls (given olive oil only). We also found a dose dependent improvement of plasma SOD levels and the myocardial GSH/GSSG ratio in thymoquinone terated rats, which was decreased in the controls given isoproterenol. Similar changes in the enzymes levels were reported in the studies conducted by Ebru *et al* (2008) and Alenzi *et al* (2010), mentioned above.

The study has demonstrated the usefulness of thymoquinone, a major active principle of *N. sativa* in the protection of heart against isoproterenol induced stress, which is a classical example of the excess catecholamines related myocardial hypoxia, angina, acute coronary insufficiency as well as 'stress cardiomyopathy'.

CONCLUSIONS

The results of our study have revealed an antioxidant activity and the cardioprotective effect of thymoquinone against the myocardial injury induced by isoproterenol.

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