Protective Effects of Thymoquinone on Endothelial Cell Dysfunction in Hypercholesterolemia

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ABSTRACT

Background: Increased reactive oxygen species (ROS) have been implicated as important mechanisms that contribute to endothelial dysfunction (ED). The administration of thymoquinone in animal models significantly inhibits ROS production. **Purpose:** The protective effects of thymoquinone on endothelial cell dysfunction were studied in cholesterol-fed rabbits. Thirty rabbits were randomly divided into five groups. **Methods:** The negative control group was fed a standard diet, the positive control group was fed the same diet with 2 % cholesterol, the Thymoquinone group was fed the same diet with 2 % cholesterol and Thymoquinone 100 mg/Kg BW/day, 200 mg/Kg BW/day or 400 mg/Kg BW/day. **Results:** The cholesterol-rich diet significantly increased Malondialdehyde (MDA) in the aortic blood vessels, as reflected by Thiobarbituric Acid-Reactive Substances (TBARS), inhibited endothelium-dependent vascular relaxations to acetylcholine and decrease cyclic GMP were compared with vessels from normal rabbits (negative control). In cholesterol-fed rabbits, Thymoquinone treatment decreased MDA in plasma production, improved endothelium-dependent relaxations to acetylcholine and increase cyclic GMP production. **Conclusion:** These results suggest that dietary treatment of rabbits with thymoquinone may prevent superoxide anion (O2-) induced inactivation of endothelium-dependent relaxing factor (EDRF), improve the endothelium-dependent relaxation to acetylcholine in the aortic blood vessels, and increase cyclic GMP content in aortic of cholesterol-fed rabbits.
Keywords: thymoquinone, malondialdehyde, EDRF, cyclic GMP

Received: 21-12-2023 **Revised:** 30-04-2024 **Accepted:** 30-05-2024

INTRODUCTION

The vascular endothelium is important in a number of homeostatic functions including the regulation of blood flow, vascular tone, and local platelet function (Shimokawa,1999). Endothelium-dependent relaxant effects on vascular smooth muscle are thought to be mediated by releasing endothelium-derived relaxing factor (EDRF), NO, or an NO related substance, followed by an increase in the cyclic GMP content in smooth muscle (Sausbier *et al.*,2000; Fujitani and Karaki,1993; Karaki and Sudjarwo,1993). Endothelium dependent vascular relaxations are impaired in numerous disease states, including hypercholesterolemia, atherosclerosis, hypertension, chronic heart failure, and diabetes mellitus (Shimokawa,1999; Verbeuren *et al.*,1990). Bioassay experiments have suggested that impaired synthesis or release of endothelium-derived relaxing factor might contribute to the abnormal endothelium- dependent relaxation in hypercholesterolemic animals (Stephanie and Cor, 2005). It has shown that shortterm cholesterol feeding in rabbit increases endothelial O2- production, seemingly from xanthine oxidase. Thus, there is substantial

evidence that hypercholesterolemia can impair endothelium-dependent relaxation via oxidative stress inactivation of endothelium-derived relaxing factor (Ohara *et al.*,1992; Jiang *et al.*,2001). In cholesterol-fed rabbits, antioxidant treatment normalized endothelium-dependent relaxations to acetylcholine (Sudjarwo, 2002). Administration of polyethylene glycolated superoxide dismutase (SOD) to increase vascular SOD levels improved endothelium-dependent relaxation in atherosclerotic rabbits (Siekmeier *et al.*, 2007; Valko,2007; Rui-Li *et al.*,2008). Also administration of antioxidant such as Vitamin E, Vitamin C and probucol could improve endothelium-dependent relaxation, normalized endothelial O2- production in hypercholesterolemic vessels and reduces lipid peroxidation in the plasma (Inoue and Nishida,1998; Mahfouz *et al*,,1997; Margurite *et al.*,2003).

The therapeutic role of thymoquinone in different diseases has been reported widely. Thymoquinone has also been used as a potent antidiabetic agent by lowering the concentrations of cholesterol and triglycerides, and enhancing the high-density lipoprotein, and insulin sensitivity (Pelegrin *et al.*, 2019). In addition, the intraperitoneal administration of thymoquinone in animal models significantly prevents the oxidative damage by enhancing the levels of antioxidant enzymes such as superoxide dismutase and catalase activity (Nagi *et al.*, 1999; Hosseinzadeh *et al.*, 2007). Thymoquinone also acts as an anti-inflammatory agent and prevents the expression of IL-6, IL-1_, and cyclooxygenase-2 in experimental rats. Moreover, thymoquinone significantly enhances the concentrations of antioxidant enzymes and lowers the MDA level (Umar *et al.*, 2012; Suddek, 2014) .The purpose of our studies was to investigate the protective effect of thymoquinone on endothelial cell in hypercholesterolemia.

METHODS

Animal preparation

New Zealand White rabbits 6 to 8 weeks old weighing between 1.8 and 2.0 kg, after 1 week of adaptation, were randomly divided into five groups. The negative control group was fed a standard diet, the positive control group was fed the same diet with 2 % cholesterol, the thymoquinone group was fed the same diet with 2% cholesterol and thymoquinone 100 mg/Kg BW/day, 200 mg/Kg BW/day or 400 mg/Kg BW/day. After 8 weeks of dietary treatment, The animals were euthanized by having their necks severed. Median thoracotomy was then performed, and the aorta was removed to obtain the rings for assessing endothelial function, MDA and c GMP content.

Preparations, solutions and measurement of muscle tension

The thoracic aorta was isolated from rabbits, cut into spiral strips (1-2 mm in width and 5-7 mm in length) and placed in normal physiological salt solution which contained (mM): NaCl 136.9, KCl 5.4, CaCl2 1.5, MgCl2 1.0, NaHCO3 23.8, ethylenediamine-tetraacetic acid 0.01 and glucose 5.5. A high K+ solution was made by substituting 69.6 mM NaCl with equimolar KCl. These solutions were saturated with a mixture of 95 % O2 and 5 % CO2 at 37 °C and pH 7.4. Muscle tension was recorded isometrically with a force-displacement transducer. Each muscle strip was attached to a holder under a resting tension of 1 g and equilibrated for 60-90 min in a 10-ml muscle bath until the contractile

response to the high K+ solution had become stable.

The functional integrity of the vascular endothelium was assessed by measuring whether 1 µM Acetylcholine induced almost complete relaxation in the aortas stimulated with 100 nM norepinephrine (Sudjarwo *et al.*,1992).

Measurement of TBARS levels in the aorta

Malondialdehyde (MDA) levels were measured by thiobarbituric reactive substances (TBARS) assay. The aortic samples were homogenized in cold trichloroacetic acid (TCA) (1 mg of tissue per mL of 10% TCA). After centrifugation, a portion of the supernatant was added to an equal volume of thiobarbituric acid (0.6% v/v), and the mixture was heated at 100°C for 20 minutes. The MDA concentration was calculated by use of a spectrophotometer, with absorption of 532 nm and the results were expressed in n mol/mg of dry tissue.

Measurement of cyclic GMP

Aortic strips were incubated with krebs solution containing 100 nM norepinephrine for 5 minutes. Then the strips were incubated with concentration of 1 µM acetylcholine. After 20 sec incubation, except where otherwise stated, the preparations were frozen quickly in liquid nitrogen. Aortic strips frozen in liquid nitrogen were transferred to 5 % (W/V) trichloroacetic acid solution and homogenized in a Potter glass homogenizer on ice. The homogenates were centrifuged at 1700 x g for 15 min at 4 °C. The supernatants were extracted 3 times with 3 volumes of water- saturated ether, and cyclic GMP contents were measured ELISA using a kit from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.).

Data analysis

Results were expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Effect Thymoquinone on Lipid Peroxidation in aorta

The lipid peroxidation production was 0.14 ± 0.03; 0.69 ± 0.06; 0.73 ± 0.4; 0.43 ± 0.07, 0.28 ± 0.05 n mol/mg protein in negative control, positive control, treatment thymoquinone at dose 100 mg/Kg BW, 200 mg/Kg BW and dose 400 mg/Kg BW, respectively. In the positive control (hypercholesterolemic) group, the level of TBARS was significantly increased compared to negative control group ($p<0.05$). Treatment with thymoquinone at dose 200 mg/Kg BW and 400 mg/Kg BW but not at dose 100 mg/Kg BW markedly reduced aorta TBARS in hypercholesterolemia which was significantly different from the positive control ($p<0.05$) (Figure 1).

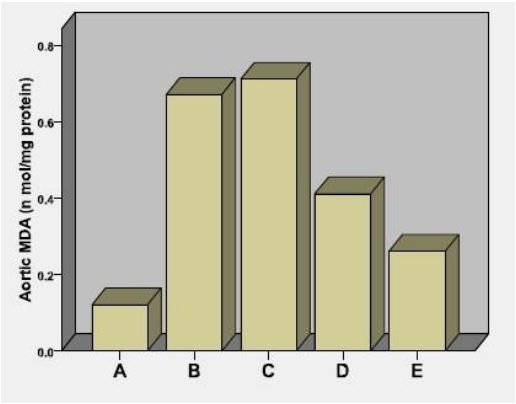

Figure 1 Bar graph showing plasma lipid peroxidation (Malondialdehyde) as determined by thiobarbituric reactive substances (TBARS). Negative control (A), Positive control (B), thymoquinone treatment at dose 100 mg/Kg BW (C), 200 mg/Kg BW

(D), and thymoquinone treatment at dose 400 mg/Kg BW (E). Each point represents the mean of six experiments.

Effect of Thymoquinone on Acetylcholine induced endhothelium - dependent vasorelaxation

Table 1. Effect of thymoquinone on acetylcholine-induced endothelium-dependent vasorelaxation

Group	Vesorelaxation		
	10nm	**100nm**	**1µm**
Positive control	21.3±2.1[a]	70.4±5.2 [a]	85.3±6.2[a]
Thymoquinone 100 mg/kg BW	7.3±1.2 [b]	46.7±2.3 [b]	59.2±2.3[b]
Thymoquinone 200 mg/kg BW	8.3±1.9 [b]	49.3±3.8 [b]	61.7±4.1[b]
Thymoquinone 400 mg/kg BW	14.6±1.7 [c]	61.2±3.5 [c]	75.1±4.6[c]

[a-c] A significant difference between the means is indicated by a distinct superscript in each column ($p < 0.05$).

Table 1 shows the concentration-response for the relaxant effect of acetylcholine in the norepinephrine-stimulated aorta. Endothelium-dependent relaxation evoked by acetylcholine was significantly impaired in the aortic ring from the cholesterol-fed (positive control) group as compared to those in the negative control group ($p<0.05$). The aorta from hypercholesterolemic rabbits treated with thymoquinone at doses of 200 mg/Kg BW and 400 mg/Kg BW but not at a dose 100 mg/Kg BW showed marked improvement of the impaired endothelium-dependent relaxation which was significantly different from positive control group ($p<0.05$).

Effect of thymoquinone on Acetylcholine-induced c GMP increase

The cyclic GMP production was 29.4 ± 2.8, 16.7 ± 1.9, 17.4 ± 1.5, 21.5 ± 2.1 and 25.7 ± 1.6 f mol/µg in negative control positive control, treatment thymoquinone at dose 100 mg/kg BW, dose 200 mg/Kg BW and dose 400 mg/KgBW, respectively. In the positive control (hypercholesterolemic) group, the cyclic GMP production was significantly decreased compared to the negative control group ($p<0.05$). The treatment with thymoquinone at dose 200 mg/Kg BW and 400 mg/KgBW but not at dose 100 mg/Kg BW markedly increase cyclic GMP production in hypercholesterolemia which was significantly different from the positive control ($p<0.05$) (Figure 2).

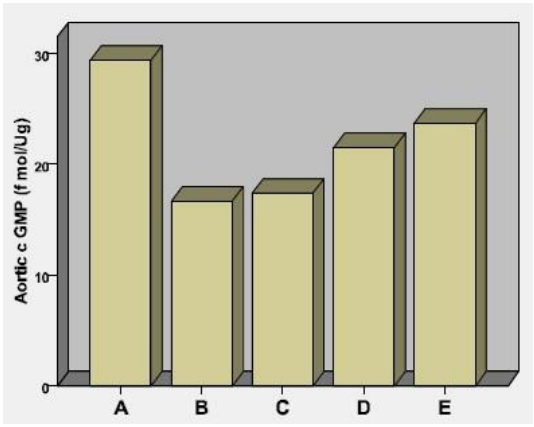

Figure 2 Effect of thymoquinone at dose 100 mg/Kg BW, dose 200 mg/kgBW and 400 mg/Kg BW on the increase in the cyclic GMP content in rabbits aortic strips after stimulation with 1 µM acetylcholine. Negative control (A), Positive control (B), thymoquinone treatment at dose 100 mg/Kg BW (C), thymoquinone treatment at dose 200 mg/Kg BW (D), and thymoquinone treatment at dose 100 mg/Kg BW (E). Each columns

represents the mean of six experiments and SEM, $p<0.05$.

In the present study, we demonstrated that in the hypercholesterolemic rabbit-induced lipid peroxidation increase. This was associated with the production of aortic TBARS. In the hypercholesterolemic rabbit also induced cyclic GMP production decrease and impaired endothelium-dependent relaxation. This is consistent with previous observations that in the hypercholesterolemic rabbit and pig are associated with impairments of endothelium-dependent relaxation and is due, at least in part, to reduced production of EDRF and cyclic GMP by endothelial cells (Fujitani *et al*,1993; Jiang *et al.*, 2000). In addition, the blunted endothelium-dependent relaxation in hypercholesterolemic animals may also result from the destruction of EDRF by superoxide anion (Inoue *et al.*, 1998). The antioxidant such as beta carotene, alpha tocopherol and probucol have been reported to improve endothelium–depend ent relaxation in hypercolesterolemic rabbits, suggesting that the free radical scavenging property of these antioxidants might play an important role in the protective effect on endothelial dysfunction (Mahfouz *et al.*,1997; Margurite *et al.*, 2003). Recently, it has been reported that thymoquinone has potent antioxidant, inhibits oxidation of low density lipoprotein and inhibit lipid peroxidation in vitro effect (Umar *et al.*, 2012; Hosseinzadeh *et al.*, 2007). In our experiments, we also obtain several results indicating that this may be case: 1) in the hypercholesterolemic rabbits significantly inhibited acetylcholine - induced endothelium-dependent relaxation, increase lipid peroxidation (malondialdehyde) and decreased cyclic GMP production, 2) the treatment with thymoquinone in hypercholesterolemic rabbits significantly reduced lipid peroxidation (malondialdehyde) production, augmented acetylcholine-induced endothelium-dependent relaxation and increased cyclic GMP production. These results suggest that dietary treatment of rabbits with thymoquinone may prevent superoxide anion (O2-) induced inactivation of endothelium-dependent relaxing factor (EDRF), improve the endothelium-dependent relaxation to acetylcholine in the aortic blood vessels, and increase cyclic GMP content in aortic of cholesterol-fed rabbits.

CONCLUSION

Thymoquinone not only improves endothelium-dependent relaxations but also reduces lipid peroxidation (malondialdehyde) in the aorta and enhanced the tissue content cycclic GMP in hypercholesterolemic rabbits. These findings suggest that thymoquinone might play an important role in the protective effect of endothelial dysfunction in hypercholesterolemia.

Acknowledgement

The authors are thankful to Program Study of Pharmacy, Faculty of Medicine, Hang Tuah University, Surabaya for providing support and necessary facilities to carry out the research work.

Author Contribution

GW conceptualization, contributed to the animal study, analyzed data and draft preparation, writing review, and editing.

Competing Interest

The authors declare that there is no conflict of interest.

Ethical Approval

All trials were examined and approved by the Faculty of Medicine at

Hang Tuah University's Ethical Approval Committee assessed this work, and it was given ethical approval under No. 9.UHT.132.05.2023.

Data Availability

The article includes data that was used to support the study's conclusions.

REFERENCES

Al-Oanzi, Z.H., Alenazy, F.O., Alhassan, H.H., El-Aassar, M.R., Alzarea, A.I., Alzarea, S.I., Abbas, A.M., Alanazi, M.H. and Al-Enazi, M.M., 2022. Effects of Thymoquinone Alone or in Combination with Losartan on the Cardiotoxicity Caused by Oxidative Stress and Inflammation in Hypercholesterolemia. Journal of Cardiovascular Development and Disease, 9(12), p.428.

Ali, S., Hashim, A., Shiekh, A., Majid, S. and Rehman, M.U., 2022. The cardioprotective effect of thymoquinone from Nigella sativa. In Black Seeds (Nigella Sativa) (pp. 239-252). Elsevier.

Forouzanfar, F. and Hosseinzadeh, H., 2020. Protective role of Nigella sativa and thymoquinone in oxidative stress: a review. Nuts and seeds in health and disease prevention, pp.127-146.

Fujitani, Y., Ueda, H., Okada, T., Urade, Y., Karaki H. 1993. A selective agonist of endothelin type B receptors, IRL 1620, stimulates cyclic GMP increase via nitric oxide formation in rat aorta. Journal of Pharmacology and Experimental Therapeutics. 267: 683-9.

Hosseinzadeh, H., Parvardeh, S., Asl, M.N., Sadeghnia, H.R., Ziaee, T. 2007. Effect of thymoquinone and Nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. Phytomedicine, 14: 621–627.

Inoue, N., Ohara, Y., Fukai, T., Harrison, D.G., Nishida, K. 1998. Probucol improves endothelial-dependent relaxation and decreases vascular superoxide production in cholesterol-fed rabbits. American Journal of the Medical Sciences. 242-7.

Jiang, F., Gibson, A.P., Dusting, G.J. 2001. Endothelial dysfunction induced by oxidized low-density lipoproteins in isolated mouse aorta: a comparison with apolipoprotein-E deficient mice. European Journal of Pharmacology. 424:141-149.

Jiang, J.H,, Valen, G., Tokuno, S., Thoren, P., Pernow, J. 2000. Endothelial dysfunction in atherosclerotic mice: improved relaxation by combined supplementation with L-arginine-tetrahydrobiopterin and enhanced vasoconstriction by endothelin. British Journal of Pharmacology. 131:1255-1261

Karaki, H., Sudjarwo, S.A., Hori, M, Takai M, Urade Y and Okada T. Induction of endothelium dependent relaxation in the rat aorta by IRL 1620, a novel and selective agonist at the endothelin ETB receptor. British Journal of Pharmacology. 1993; 109: 371-4.

Leong, X.F., Choy, K.W. and Alias, A., 2021. Anti-inflammatory effects of thymoquinone in atherosclerosis: A mini review. Frontiers in pharmacology, 12, p.758929.

Li, W., Zhang, H., Zhang, L., Zhang, T. and Ding, H., 2022. Effect of Thymoquinone on Renal Damage Induced by Hyperlipidemia in LDL Receptor-Deficient (LDL-R-/-) Mice. BioMed Research International, 2022.

Mahfouz, M.M., Kawano, H., Kummerow., F.A. 1997. Effect of cholesterol rich diets with and

without added vitamin E and C on them severity of the atherosclerosis in rabbits.The American Journal of Clinical Nutrition. 6:1240-9.

Majdalawieh, A.F., Yousef, S.M. and Abu-Yousef, I.A., 2021. Thymoquinone, a major constituent in Nigella sativa seeds, is a potential preventative and treatment option for atherosclerosis. European Journal of Pharmacology, 909, p.174420.

Nagi, M.N., Alam, K., Badary, O.A., Al-Shabanah, O.A., Al-Sawaf, H.A., Al-Bekairi, A.M. 1999. Thymoquinone protects against carbon tetrachloride hepatotoxicity in mice via an antioxidant mechanism. Biochemistry and Molecular Biology International. 47: 153-159.

Pei, Z.W., Guo, Y., Zhu, H.L., Dong, M., Zhang, Q. and Wang, F., 2020. Thymoquinone protects against hyperlipidemia-induced cardiac damage in low-density lipoprotein receptor-deficient (LDL-R-/-) mice via its anti-inflammatory and antipyroptotic effects. BioMed Research International, 2020.

Pelegrin, S., Galtier, F., Chalançon, A., Gagnol, J.P., Barbanel, A.M., Pélissier, Y., Larroque, M., Lepape, S., Faucanié, M., Gabillaud, I. 2019. Effects of Nigella sativa seeds (black cumin) on insulin secretion and lipid profile: A pilot study in healthy volunteers. British. Journal of Clinical Pharmacology. 85:1607-1611.

Ragheb, A., Attia, A., Elbarbry, F., Prasad, K. and Shoker, A., 2011. Attenuated combined action of cyclosporine A and hyperlipidemia on atherogenesis in rabbits by thymoquinone. Evidence-Based Complementary and Alternative Medicine, 2011.

Suddek., G.M. 2014. Protective role of thymoquinone against liver damage induced by tamoxifen in female rats. Canadian Journal of Physiology and Pharmacology. 92: 640-644.

Umar, S., Zargan, J., Umar, K., Ahmad, S., Katiyar, C.K., Khan, H.A. 2012. Modulation of the oxidative stress and inflammatory cytokine response by thymoquinone in the collagen induced arthritis in Wistar rats. Chemical Interactions. 197, 40-46.

Xu, J., Zhu, L., Liu, H., Li, M., Liu, Y., Yang, F. and Pei, Z., 2018. Thymoquinone reduces cardiac damage caused by hypercholesterolemia in apolipoprotein E-deficient mice. Lipids in health and disease, 17, pp.1-9.

Zhang, L., Zhang, H., Ma, J., Wang, Y., Pei, Z. and Ding, H., 2022. Effects of thymoquinone against angiotensin II-induced cardiac damage in apolipoprotein E-deficient mice. International Journal of Molecular Medicine, 49(5), pp.1-12.

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