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Research Article

Cardioprotective Effects of Thymoquinone on Myocardial Fibrosis

Saugi Abduh[1,2,*], Purwanto Bambang[3], Dirgahayu Paramasari[4], Soetrisno[5]

ABSTRACT

Introduction: Thymoquinone (TQ) is one of the active ingredients in herbal plants such as *Nigella sativa* which has antioxidant and anti-inflammatory properties thus may inhibits cardiac fibrosis formation. This study aims to determine the effectiveness of Thymoquinone as a cardioprotective agent in suppressing the extent of fibrosis in Wistar rats induced with lipopolysaccharide (LPS). **Methods:** This post-test only control study used 30 Wistar rats which were divided into 5 groups: saline, LPS-induced cardiac fibrosis, LPS-induced cardiac fibrosis treated with TQ 10 mg/mL, LPS-induced cardiac fibrosis treated with TQ 20 mg/mL, and LPS-induced cardiac fibrosis treated with TQ 40 mg/mL. Serum IL-6, GSH, and cTnT levels were measured using ELISA, and Mason's trichrome staining was used to assess myocardial fibrosis. **Results:** The LPS10+TQ20 and LPS10+TQ40 groups exhibited significantly lower levels of IL-6 compared to the LPS10+TQ10 group ($p < 0.05$). GSH levels did not show a significant decrease in the TQ groups across different doses ($p=0.771$). The TQ-treated group demonstrated lower cTnT levels compared to the LPS-only group ($p<0.05$). Thymoquinone treatment resulted in reduced fibrosis area compared to the LPS10 group ($p<0.05$). **Conclusions:** TQ has a promising cardioprotective effect on the formation of cardiac fibrosis in Wistar rats induced with LPS.

Key words: Lipopolysaccharide, *Nigella Sativa*, Cardiac Fibrosis, Oxidative Stress.

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INTRODUCTION

Myocardial fibrosis is a sequele of sustained inflammation after myocardial infarction. Lipopolysaccharide (LPS) inducing systemic inflammation, oxidative stress, and cardiomyocyte apoptosis.[1] LPS triggers the production of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, *via* various signaling pathways such as MAPKs pathway, phosphoinositide-3-kinase pathway (PI3K/Akt), signal transducer and activator of transcription-3 (STAT3), and glycogen synthase kinase-3 (GSK3) pathways.[2] LPS also induces reactive oxygen species formation leading to oxidative stress and reducing GSH levels.[3] The imbalance between pro-oxidants and antioxidants can activate apoptosis pathways and release markers such as cardiac troponin T (cTnT).[4] Myocardial fibrosis induced by increased TGF-β levels due to prolonged inflammation and oxidative stress, further impairs cardiac function.[5]

Thymoquinone (TQ) is a polyphenol found in *Nigella Sativa* which has anti-carcinogenic, anti-inflammatory, and antioxidant properties.

The administration of TQ can inhibit the production of NFkB induced by LPS.[6] The decrease in NF-kB activity will cause a decrease in proinflammatory cytokines and oxidative stress, so that the formation of fibrosis in the heart can be prevented.[7] Previous research stated that the administration of TQ in LPS-induced rats caused a significant decrease in TNF-α levels and an increase in SOD levels compared to rats receiving only LPS.[8] Previous research also stated that TQ can prevent myocardial and perivascular fibrosis by suppressing chronic inflammation and oxidative stress. Research on the effectiveness of TQ in various doses to prevent heart fibrosis has not been widely conducted. This study aims to determine the effectiveness of TQ in various doses as cardioprotective in LPS-induced rats.

MATERIALS AND METHODS

Design

This experimental study used a posttest only control group design conducted in April 2022. The research samples are Wistar rat strains that are maintained in the Intergrated Biomedicine Laboratory of Medical Faculty of Sultan Agung University. This study has passed the ethical review by the Ethics Committee of Medical Faculty of Sultan Agung University (*No. 395/X/2022/Komisi Bioetik*).

Animal model

The animal model for myocardial fibrosis was done by administering LPS 10 mg/mL/hour intraperitoneally for 14 consecutive days. Twenty-four male Wistar rats weighted ±200g were then randomized and divided into 5 groups with 6 rats in each group, namely: (A) Saline group ;(B) Lipopolysaccharide 10 mg/mL group; (C) Lipopolysaccharide 10 mg/mL + Thymoquinone 10 mg/kgBW group; (D) Lipopolysaccharide 10 mg/mL + Thymoquinone 20 mg/kgBW group; (E) Lipopolysaccharide 10 mg/mL + Thymoquinone 40 mg/kgBW.Rats in the negative control group were only given normal saline. Termination was performed on the 15th day by inhalation of chloroform and blood samples were taken from the orbital sinus and placed in EDTA tubes, then centrifuged at a speed of 3000 rpm for 20 minutes, then the serum was taken and stored at -70^{0}C. The heart samples were taken and fixed using Bouin's solution (75 ml picric acid, 25 ml formaldehyde, 5 ml acetic acid). The measured variables were GSH, IL-6, cardiac troponin T (cTNT), and the area of fibrosis.

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Measurement of IL-6, GSH, and cTnT

The levels of IL-6, GSH, and cTnT in serum were measured spectrophotometrically using an ELISA kit (Bioenzy, Indonesia) according to the manufacturer's instructions.

Histopathological assessment

The hearts were taken and fixed using Bouin's solution for 24 hours before preparation according to standard protocols. The samples were cut to a thickness of 5 µm, and then stained with Masson's Trichrome stain by a pathologist. The preparation was observed under a binocular microscope (magnification 40x). The histopathological preparation was assessed by two pathologists in separate locations under light microscope.

Statistical analysis

The data for GSH, IL-6, cTNT, and the area of fibrosis are presented descriptively. Normality test was conducted using Shapiro-Wilk test. Homogeneity test was conducted using Levene's test. Data with normal distribution and homogeneity were tested using one-way ANOVA to determine differences between groups. Data with non-normal distribution were tested using Kruskal Wallis test. The data obtained were processed using computerized methods, and the analysis was performed using SPSS 25.0 for Windows.

RESULTS

Serum IL-6 level

The measurement showed a significant difference in serum IL-6 levels in all groups (Figure 1A). The highest level was found in the LPS 10+TQ 10 group, which was 69.96±0.02 (pg/ml). The lowest level was found in the LPS 10 + TQ 40 group, which was 60.64±2.29 (pg/ml). Posthoc analysis showed a significant difference in IL-6 level between the LPS10+TQ10 group and both the LPS10+TQ20 and LPS10+TQ40 groups, but there was no significant difference in IL-6 level between the LPS10+TQ20 and LPS10+TQ40 groups. Interestingly, the LPS10 + TQ10 group had a higher serum IL-6 level compared to the LPS10 group.

Serum GSH level

The results showed no significant difference in serum GSH levels between groups receiving only lipopolysaccharide and both lipopolysaccharide and thymoquinone, where the highest level was found in the LPS 10 group at 159.00±3.05 (Figure 2). However, groups receiving TQ had lower serum GSH levels at 147.33±3.92 (pg/ml); 152.66±7.53 (pg/ml); and 148.33±14.67 (pg/ml) in the LPS10 + TQ10, LPS10 + TQ20, and LPS10 + TQ40 groups, respectively.

Serum cTnT level

The result showed that the group treated with thymoquinone had a significantly lower level of serum cTnT, with the highest levels found in the LPS 10 group at 33.84±2.38 ng/dL (Figure 3). The lowest cTnT levels were found in the LPS10+TQ20 group at 28.45±2.83 ng/dL ($p<0.05$). Interestingly, the LPS10+TQ40 group had higher cTnT levels than the LPS10+TQ10 and LPS10+TQ20 groups at 31.26±0.33 ng/dL. Interestingly, the LPS10+TQ40 was not statistically different.

Myocardial fibrosis

The LPS group had the largest area of cardiac fibrosis compared to other groups (Figure 4 & 5). The groups that received TQ had smaller areas of cardiac fibrosis compared to the LPS group. The higher the dose of TQ given, the smaller the percentage of the area of cardiac fibrosis, where the smallest area of fibrosis was found in the LPS10+TQ40 group which was 7.786%.

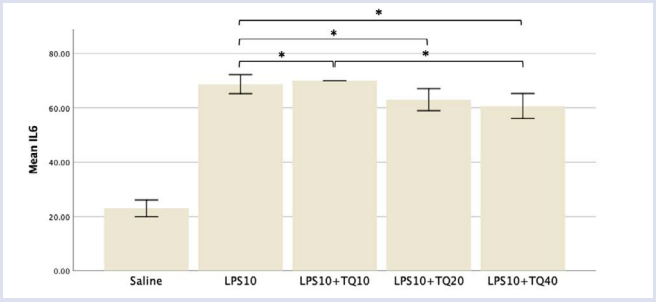

Figure 1: Analyses for IL-6 in all groups. *mean significant difference, (A) Saline group, (B) LPS10 = LPS 10 mg/mL, (C) LPS10+TQ10 = LPS 10 mg/mL + TQ 10 mg/mL, (D) LPS10+TQ20 = LPS 10 mg/mL + TQ 20 mg/mL, (E) LPS10+TQ40 = LPS 10 mg/mL + TQ 40 mg/mL

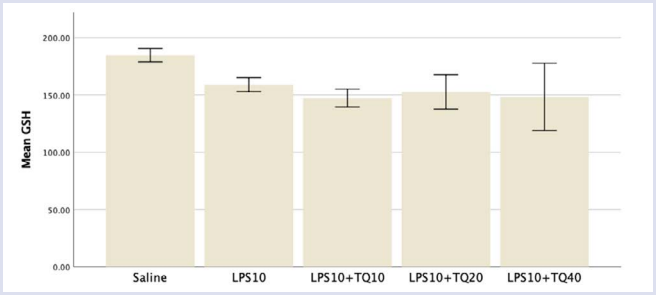

Figure 2: Analyses for GSH in all groups. *mean significant difference, (A) Saline group, (B) LPS10 = LPS 10 mg/mL, (C) LPS10+TQ10 = LPS 10 mg/mL + TQ 10 mg/mL, (D) LPS10+TQ20 = LPS 10 mg/mL + TQ 20 mg/mL, (E) LPS10+TQ40 = LPS 10 mg/mL + TQ 40 mg/mL

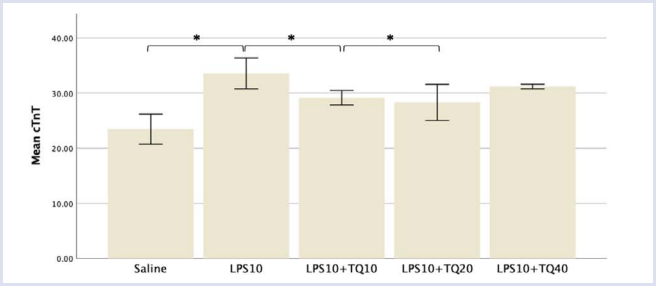

Figure 3: Analyses for cTnT in all groups. *mean significant difference, (A) Saline group, (B) LPS10 = LPS 10 mg/mL, (C) LPS10+TQ10 = LPS 10 mg/mL + TQ 10 mg/mL, (D) LPS10+TQ20 = LPS 10 mg/mL + TQ 20 mg/mL, (E) LPS10+TQ40 = LPS 10 mg/mL + TQ 40 mg/mL

DISCUSSION

This study demonstrates that the administration of TQ can significantly reduce the levels of IL-6. In addition, the results of this study show that the groups receiving TQ with doses of 20 mg/mL and 40 mg/mL had significantly lower serum IL-6 levels compared to the 10 mg/mL dose. This is believed to be due to TQ inhibiting the activation of NFkB by LPS, thus suppressing the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α. These results are consistent with previous studies that have reported a significant decrease in IL-6 levels in rats induced with isoproterenol given thymoquinone 20 mg/mL compared to the control group induced with isoproterenol alone without thymoquinone addition.[9]

Other findings also showed that the administration of TQ in LPS-induced rats resulted in a non-significant decrease in GSH levels

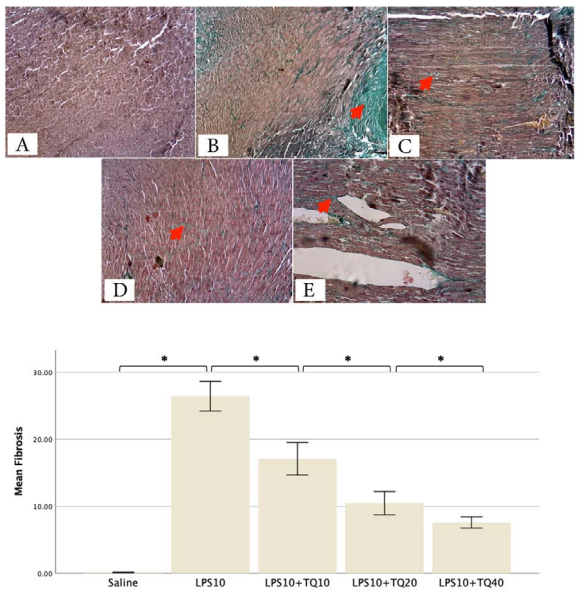

Figure 4: Mason's trichrome staining analyses for GSH in all groups. *mean significant difference, (A)Saline group, (B) LPS10 = LPS 10 mg/mL, (C) LPS10+TQ10 = LPS 10 mg/mL + TQ 10 mg/mL, (D) LPS10+TQ20 = LPS 10 mg/mL + TQ 20 mg/mL, (E) LPS10+TQ40 = LPS 10 mg/mL + TQ 40 mg/mL

compared to the group that only received LPS induction. This is believed to be due to the antioxidant effect of TQ in neutralizing ROS accumulation in tissues due to LPS induction being less optimal due to the higher dose of LPS used in this study. These results are not consistent with previous studies that have reported a significant increase in GSH levels in rats with liver dysfunction due to LPS treated with thymoquinone compared to untreated group.[3]

Furthermore, the administration of TQ to LPS-induced mice can lead to a significant decrease in cTnT levels compared to the group that only received LPS induction. This is believed to be due to the protective effect of TQ in suppressing excessive oxidative stress and inflammation caused by LPS induction, which inhibits cardiomyocyte death and rupture of the cell membrane, causing this protein to leak into the extracellular compartment. These findings are consistent with previous research that found a significant decrease in troponin I levels in the group given thymoquinone at a dose of 20 mg/mL compared to the control group induced with isoproterenol.[10] Interestingly, the LPS+TQ20 group had lower cTnT levels than the LPS10+TQ10 group, but in the LPS+TQ40 group, troponin I levels increased compared to the LPS10+TQ20 and LPS10+TQ10 groups. These findings reject the hypothesis that increasing thymoquinone dose is consistent with decreasing troponin I levels in LPS-induced mice. Previous studies stated that excessive doses of thymoquinone do not increase its protective effect, which can be due to excessive antioxidant effects where excessive antioxidants usage at high concentrations can cause the antioxidant effect to disappear and even become pro-oxidants.[11] Previous study also stated an increase in troponin levels in patients with chronic kidney disease without a history of acute coronary syndrome.[12] This may explain why the group receiving a dose of 40 mg of thymoquinone had higher troponin levels than the other groups receiving lower doses of thymoquinone.

The results of this study showed that there was a narrowing of the fibrosis area in the heart in the group that received TQ induced with LPS compared to the group that only received LPS. This study also showed that the larger the dose of TQ received, the smaller the fibrosis area that formed. The smallest fibrosis area formed in this study was in the group that received a dose of 40 mg/mL TQ. This certainly supports the hypothesis that an increase in thymoquinone dose is accompanied by a decrease in the size of the heart fibrosis area in LPS-induced rats due to TQ's ability to suppress the production of proinflammatory cytokines, thus preventing ongoing inflammation.[13] Proinflammatory cytokines such as IL-1, IL-6, and TNF-α can activate myofibroblasts to produce type I collagen, which is an extracellular matrix found in fibrotic tissue.[14] These findings are consistent with previous studies that have shown that the larger the dose of TQ received in LPS-induced rats, the smaller the fibrosis area that forms.[8]

The limitation of this study is the lack of comparison of the effectiveness of TQ on IL-6 levels, GSH, Troponin, and the area of fibrosis compared to the gold standard therapy for cardiac fibrosis.

CONCLUSION

TQ has a cardioprotective effect by suppressing the formation of fibrosis. The fibrosis formation is influenced by the size of the induction of TQ dose-dependently. The higher the dose of TQ given, the smaller the extent of fibrosis area formed. Further research is needed on different models to compare the effectiveness of TQ administration with the gold standard treatment currently used for heart fibrosis.

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