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THYMOQUINONE AMELIORATES CHEMOTHERAPY-INDUCED COGNITIVE IMPAIRMENT (CHEMOBRAIN) BY INHIBITING OXIDATIVE STRESS, INFLAMMATORY RESPONSE IN THE BRAIN OF RAT

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Abstract

The aim of this study was to investigate the therapeutic efficacy of antioxidant thymoquinone (TQ) treatment on neuroinflammation against the negative effects of Doxorubicin (DOX) used in cancer treatment on learning and memory by behavioural and molecular mechanisms. Forty male Wistar rats were divided into four groups: Control, DOX, TQ and DOX+TQ. DOX and DOX+TQ groups were injected with 3 mg/kg (i.p.) DOX for 4 weeks. TQ and DOX+TQ groups received 10 mg/kg TQ by gavage. Learning, locomotor activity, oxidative stress and inflammatory levels were evaluated. In behavioural experiments data, a significant decrease was observed in DOX group compared to Control group, while a significant increase was observed in DOX+TQ group compared to DOX group. Compared to the control group, TOC, IL-1β, IL-6 and TNF-α levels increased, while TAC level decreased in the DOX group. With TQ treatment, a significant decrease was observed in TOC, IL-1β, IL-6 and TNF-α levels in the TQ and DOX+TQ groups compared to the DOX group, while significant increases were observed in TAC level. In our study, DOX toxicity increased oxidative stress and neuroinflammation and caused learning and memory loss, while TQ decreased oxidative stress and neuroinflammation and improved learning and memory disorders.

Key words: doxorubicin, thymoquinone, learning and memory

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Introduction. Chemotherapy in the treatment of cancer offers hope for patients, but patients often struggle with some of the side effects of chemotherapeutic drugs [1]. Doxorubicin (DOX) is widely used clinically in cancer treatment, but its use is limited due to toxic side effects such as dose-dependent cognitive impairment, cardiomyopathy and peripheral neuropathy [2]. The antitumour mechanism of DOX is attributed to its intercalation into DNA and overproduction of reactive oxygen species (ROS) [3]. It is known that oxidized ApoA-I is unable to suppress TNF-α release and this leads to high plasma TNF-α levels [3]. TNF-α activates microglia in the brain, leading to an increase in TNF-α. TNF-α also increases NO production by glia, causing mitochondrial dysfunction, nitrosative and oxidative stress, endoplasmic reticulum stress and neuronal apoptosis [3].

Thymoquinone (TQ) a potent antioxidant, decreases the production of superoxide radicals and lipid peroxidation and increases the activities of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GSH), glutathione reductase and superoxide dismutase (SOD) [4]. Furthermore, TQ ameliorated learning and memory function in animal models of Alzheimer's disease thanks to its anti-neurodegenerative properties [5]. This study investigates the therapeutic efficacy of TQ against DOX-induced learning and memory impairment, oxidative and inflammatory damage.

Materials and methods. ***Ethics statement.*** All animal use and experimental protocols were approved and implemented by the Animal Care and Ethics Committee of the Akdeniz University School of Medicine (24.01.2018/protocol No. 21).

Animals and experimental grouping. In the study, 40 male Wistar Albino rats weighing 250–300 g, 12 weeks old, were used. Rats were randomized and divided into four groups: Control, TQ, DOX and DOX+TQ ($n = 10$ per group) (Fig. 1). Gavage and injection administration was started on the same day in all rats. TQ was first dissolved in Dimethyl sulphoxide (DMSO) and then mixed with saline. The solvent solution consisted of 25% DMSO and 75% saline [6].

Control Group: The rats were injected with saline (i.p.) 5 times every other day and the solution was given by gavage once daily for 4 weeks.

TQ Group: The rats were injected with saline (i.p.) 5 times every other day. TQ (10 mg/kg) was administered by gavage at 24 h intervals for 4 weeks.

DOX Group: The rats were injected with 3 mg/kg DOX (i.p.) 5 times every other day. In addition, carrier solution was administered by gavage at 24 h intervals for 4 weeks.

DOX+TQ Group: Rats were injected with 3 mg/kg DOX (i.p.) 5 times every other day. TQ (10 mg/kg) was administered by gavage at 24 h intervals for 4 weeks.

Locomotor activation was assessed in the open field test, learning and memory function was evaluated by novel object recognition (NOR) test and Y-maze test. In addition, anti/oxidant parameters (total antioxidant capacity (TAC) and total

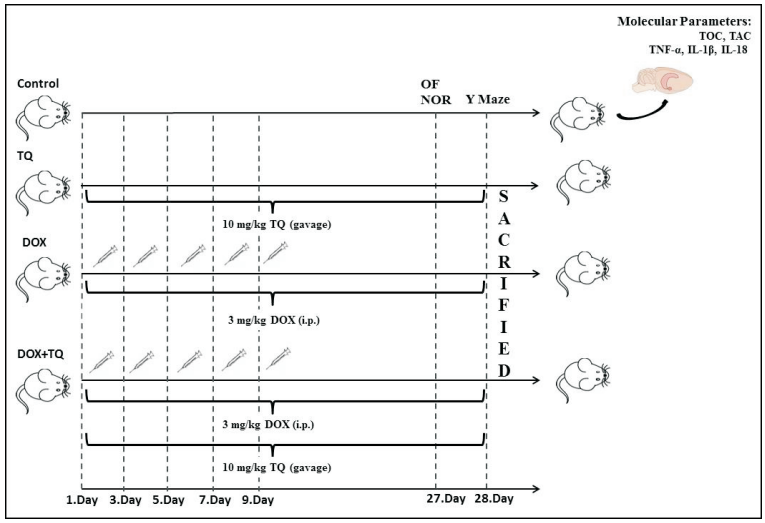


Fig. 1. Experimental protocol

oxidant capacity (TOC)) was measured in hippocampus tissue for biochemical evaluation. Neuroinflammation was evaluated by measuring TNF-α, IL-1β and IL-18 levels by ELISA method.

Behavioural experiments: Assessment of locomotors activity in the open field (OF) test. Locomotor activity was carried out in a setup with a base of 80 × 80 cm and a wall height of 40 cm. For rats to explore the apparatus, they were placed in the centre of the field and monitored and recorded by video for 5 min. Total distance travelled (cm) was calculated to evaluate locomotor activity [7].

Assessment of recognition memory in the novel object recognition (NOR) test. The Novel Object Recognition Test (NOR) test is often preferred for measuring attention or short-term memory activities [7]. In the NOR test, the discrimination index and the time spent in the novel object (s) values will be analyzed. Discrimination Index = ((Time Spent at Novel Object-Time Spent at Old Object)/Total Time) × 100.

Assessment of spatial memory in the Y-maze test. Y-maze test is used to investigate short-term memory and spatial memory. The Y-maze test consists of two stages. In the first phase of the experiment, the rats are left at the end of the starting arm and each rat is given 15 min to freely examine the other arms with the new arm fully closed. At the end of the session, the rats are removed from the maze and after one hour the arms are opened, allowing the rats to freely explore all three arms for 5 min. In this second session, subjects with spatial memory are expected to make the first turn into the "novel arm" and spend more time exploring this arm. The number of new arm entries and the time spent exploring the new arm were recorded [7].

Tissue collection. Animals were killed by decapitation on day 28 after the behavioural experiments. For biochemical analysis, hippocampus tissue was dissected from the brain and stored frozen at $-80\,^{\circ}$C. Tissues were homogenized in phosphate buffered saline (PBS, pH 7.4) centrifuged at 12 000 rpm for 20 min at $4\,^{\circ}$C and supernatants were used for biochemical analyses.

Biochemical analysis. Determining total antioxidant capacity. Total Antioxidant Capacity (TAC) in hippocampus tissues was evaluated using the OxiSelect™Total Antioxidant Capacity (TAC) Assay kit (Cell Biolabs, Inc., San Diego, CA, USA). The TAC assay is based on the reduction of copper (II) to copper (I) by antioxidants such as uric acid and the reaction with a chromogen, determining the absorbance at 490 nm. TAC levels were expressed as nmol/mg protein.

Determining total oxidant capacity. Total Oxidant Capacity (TOC) determination in hippocampus tissues was measured using the OxiSelect™In Vitro ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA). This method is based on the oxidation of dichlorodihydrofluorescin (DCFH) by ROS/RNS within the samples into the fluorescent form $2'$, $7'$-dichlorodihydrofluorescin (DCF). Samples were measured fluorometrically against a hydrogen peroxide or DCF standard at 480 nm excitation and 530 nm emission. The free radical content of oxidant molecules present in the sample was determined by comparison with a predetermined DCF or hydrogen peroxide standard curve, and concentrations were calculated by linear regression.

Enzyme-linked immunosorbent assay (ELISA). The levels of TNFα, IL-1β and IL-18 were quantified using enzyme-linked immunosorbent assay (ELISA) kits. Commercially available ELISA kits (R&D Systems, Minneapolis, USA) for rat TNF-α (EK710127), IL-1β (EK710260) and IL-18 (EK710281) were performed according to the manufacturer's instructions. TNF-α, IL-1β and IL-18 concentrations in the samples were calculated from their corresponding absorbance values via the standard curve. Data were normalized to total tissue protein and expressed as pg·mg^{-1} tissue protein.

Protein measurements. Protein concentrations were measured in the hippocampus tissues at 595 nm by a modified Bradford assay using Coomassie Plus reagent with bovine serum albumin standard (Pierce Chemical Company, Rockford, IL, USA).

Statistical analysis. The SPSS software package 20.0 program was used for all analyses. The results were given as mean ± standard error of the mean (SEM). P values less than 0.05 were considered significant. The one-way ANOVA test was used in the analysis of data with normality condition in the evaluation made with the Shapiro–Wilk test. The Tukey test was used for post-hoc analysis.

Results. ***Effect of tDCS on locomotor activity, spatial learning and memory functions.*** Total distance in the OF was significantly decreased in the DOX group compared to the control group ($p = 0.026$) (Fig. 2A). After

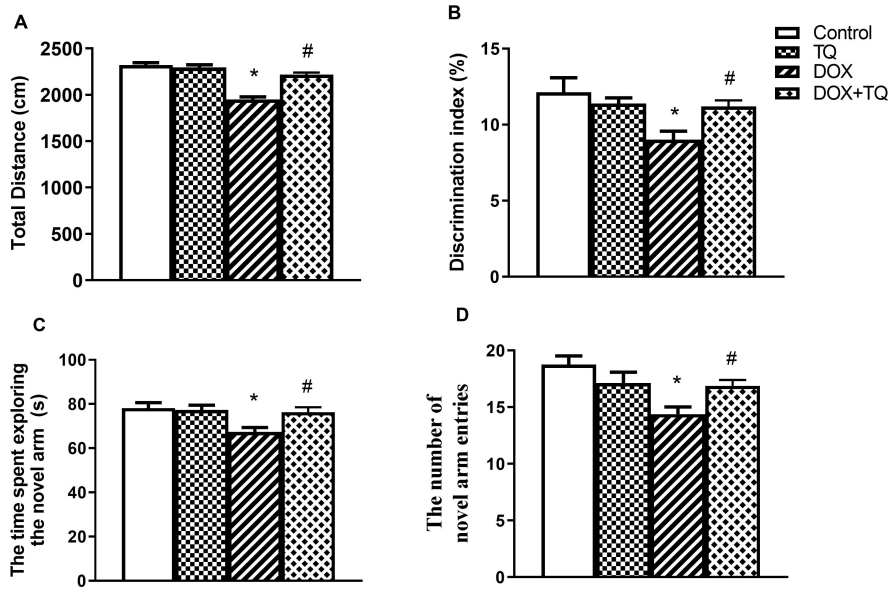


Fig. 2. Behavioural results of experimental groups. A) Total distance (cm) in OF; B) Discrimination index in NOR; C) Exploration time to the novel arm (s) in Y Maze; D) Frequency of entrance to the novel arm in Y Maze. ($n = 10$, for each group; $^{*}p < .05$ shows the difference compared to the Control group, $^{\#}p < .05$ shows the difference compared to the DOX group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM

TQ treatment, a significant increase in locomotor activity was observed in the DOX+TQ group compared to the DOX group ($p = 0.038$) (Fig. 2A). While there was a significant decrease in the learning of the DOX group compared to the control group ($p = 0.017$), there was a significant increase in the DOX+TQ group compared to the DOX group ($p = 0.034$) (Fig. 2B). It was found that there was a significant decrease the exploration time to the novel arm and the frequency of entrance to the novel arm in the DOX group compared to the control group ($p = 0.039$, $p = 0.031$), and there was a significant increase in the DOX+TQ group compared to the DOX group ($p = 0.045$, $p = 0.047$) (Fig. 2C and D).

Total oxidant capacity and total antioxidant capacity level results. A significant increase in total oxidant capacity ($p = 0.0013$) (Fig. 3A) and a significant decrease in total antioxidant capacity ($p = 0.025$) were observed in the DOX group compared to the control group (Fig. 3B). After TQ treatment, there was a significant decrease in TOC level of DOX+TQ group compared to DOX group ($p = 0.033$). On the other hand, a significant increase was found in the TAC level of the DOX+TQ group compared to the DOX group ($p = 0.032$) (Fig. 3B).

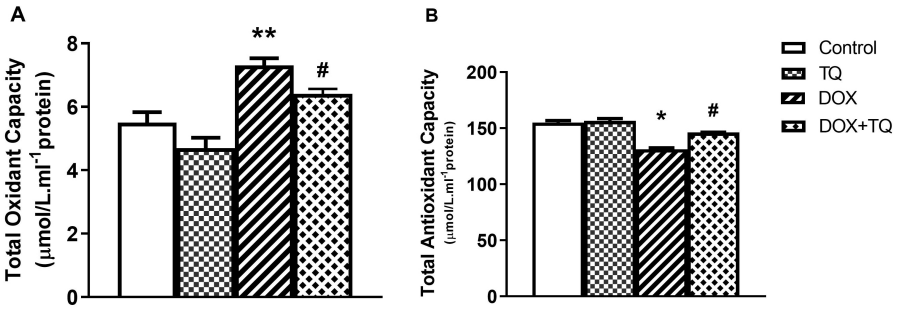


Fig. 3. Total oxidant/antioxidant capacity results. A) TOC results in hippocampus; B) TAC results in hippocampus. ($n = 10$, for each group; $^{*}p < .05$ shows the difference compared to the Control group, $^{\#}p < .05$ shows the difference compared to the DOX group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM

TNFα, IL-1β and IL-18 levels in hippocampus. TNF-α, IL-1β and IL-18 levels in the hippocampus are shown in Fig. 4. TNF-α, IL-1β and IL-18 levels of the DOX group were significantly increased compared to the control group ($p < 0.05$) (Fig. 4). TQ treatment significantly decreased all pro-inflammatory cytokine levels in DOX-induced rats ($p < 0.05$ for all).

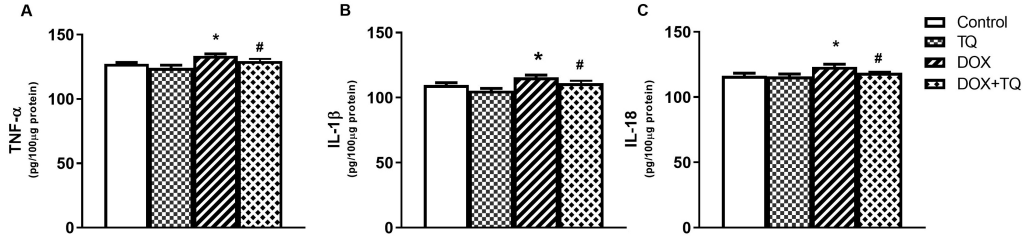


Fig. 4. Proinflammatory cytokine levels in hippocampus. A) TNF-α levels; B) IL-1β levels; C) IL-18 levels. ($n = 10$, for each group; $^{*}p < .05$ shows the difference compared to the Control group, $^{\#}p < .05$ shows the difference compared to the DOX group, one-way ANOVA test, followed by Tukey post hoc test). All data are presented as means ± SEM

Discussion. We investigated whether TQ could exert ameliorative effects on learning and memory deficit induced by DOX-induced neurotoxicity in an animal model. Furthermore, TQ increased the levels of antioxidant parameters such as TAC and decreased the level of TOC as a marker of oxidative stress. Moreover, TQ also decreased DOX-induced increased TNF-α, IL-1β and IL-18 neuroinflammation in hippocampus tissue.

DOX chemotherapy is widely used in the treatment of cancer, but it is also known to cause toxicity in vital organs such as the heart, liver and brain. DOX treatment plays a vital role in the toxicity observed in heart and brain tissue by leading to excessive ROS production and increased TNF-α levels [3]. DOX is

known to impair learning and memory in rodents [2]. We found that treatment with TQ prevented DOX-induced cognitive deficits at both neurobehavioural and hippocampal levels of oxidative stress and neuroinflammation.

TQ has therapeutic properties such as antioxidant, anti-inflammatory and anti-cancer[4]. In our study, significant improvements were observed in the learning level of the DOX+TQ treatment group compared to the DOZ group in DOX-induced learning and memory loss. Moreover, TQ treatment, especially at a dose of 10 mg/kg, significantly improved spatial memory function. Consistent with our results, Radad et al. [6] reported that TQ at doses of 15 and 7.5 mg/kg reduced learning and memory loss and had protective effects against neuronal damage in rotenone-induced Parkinson's rat model. In line with these studies, we found that TQ has strong antioxidant and neuroprotective properties against DOX-induced learning memory loss and improves learning and memory disorders.

DOX has a quinone moiety that undergoes redox cycle conversion producing reactive oxygen species (ROS) [3]. As a result of the increase in ROS, the balance between oxidants and antioxidants is disrupted, resulting in oxidative stress [3]. To cope with increased ROS production, Nrf2 functions as an antioxidant defence mechanism. Our results demonstrated that DOX-induced cognitive impairment is associated with increased oxidative stress as shown by significantly elevated TOC levels and decreased TAC levels in hippocampal tissues. Treatment with TQ can reverse the levels of these oxidative stress markers, implying that the neuroprotective effects of TQ in preventing DOX-induced cognitive impairment may be partially attributable to the antioxidant capacity of TQ.

ROS generated during the redox cycle have been reported to activate the transcription of numerous genes, including inflammatory cytokines, through activation of the redox-sensitive transcription factor NF-κB [8]. In one study, researchers observed that TNF-α was elevated after multi-agent chemotherapy DOX treatment [3]. Consistently, this study showed that DOX significantly increased the levels of TNF-α, IL-1β and IL-18 in hippocampal tissues, suggesting that DOX enhances the neuroinflammatory response in rats. In contrast, TQ treatment suppressed such an inflammatory response and decreased TNF-α, IL-1β and IL-18 levels.

Conclusions. We demonstrated that TQ is a promising neuroprotective agent that may protect against DOX-induced cognitive impairment. DOX treatment induced cognitive impairment in Wistar Albino rats and TQ was able to prevent these impairments through its anti-inflammatory, antioxidant and anti-apoptotic properties. Therefore, TQ may be a promising and potential therapeutic agent to alleviate these DOX-induced chemobrain-related deficits.

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