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Investigation of Thymoquinone's Effects on Bisphenol A-Induced Neurotoxicity, Keap-1/Nrf-2 Signalling, Oxidative Stress, and Behavior in Rats

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Citation

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Abstract

Chronic low-dose exposure to bisphenol A (BPA), a widely used environmental endocrine disruptor, has been increasingly associated with oxidative stress-mediated neurotoxicity. Thymoquinone (TQ), a bioactive constituent of *Nigella sativa*, exhibits potent antioxidant properties; however, its neuroprotective potential against BPA-induced toxicity remains incompletely understood. Given the pervasive nature of BPA exposure, identifying effective and accessible neuroprotective strategies is of growing importance. This study investigated the effects of TQ on oxidative stress, Keap-1/Nrf-2 signaling, behavior, and histopathological alterations in rats exposed to BPA. A total of 24 adult male Wistar Albino rats were randomly assigned to four groups (n = 6/group): control, BPA, BPA + 10 mg/kg TQ (BPA+TQ I), and BPA + 20 mg/kg TQ (BPA+TQ II). BPA (50 µg/kg) and TQ were administered intragastrically for 30 days. BPA exposure induced significant behavioral impairments, oxidative stress, disruption of Keap-1/Nrf-2 signaling, and marked histopathological damage in the hippocampus and cerebral cortex. TQ treatment significantly improved locomotor activity and depression-like behavior, reduced lipid peroxidation and nitric oxide levels, restored glutathione-related antioxidant capacity, and normalized Keap-1 and Nrf-2 expression ($p < 0.001$). These protective effects were more pronounced at the higher TQ dose. Taken together, these findings demonstrate that thymoquinone mitigates BPA-induced neurotoxicity through modulation of oxidative stress and the Keap-1/Nrf-2 signaling pathway, highlighting its potential as a neuroprotective agent against environmentally relevant BPA exposure.

Introduction

Bisphenol A (BPA) is a synthetic compound widely used in plastics and epoxy resins and is commonly detected in food packaging materials, water systems, medical devices, and household products[1]. Continuous environmental exposure has raised concerns regarding its potential neurotoxic effects, particularly under chronic low-dose conditions that reflect real-life human exposure.

Although regulatory agencies such as the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency have defined 50 µg/kg/day as the no-observed-adverse-effect level, emerging experimental and epidemiological evidence indicates that behavioral and cognitive alterations may occur at lower doses. Consequently, the European tolerable daily intake has been reduced to 4 µg/kg/day, emphasizing the need to investigate mechanistic effects of environmentally relevant BPA exposure[1,2].

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Oxidative stress is considered a central mechanism underlying BPA-induced neurotoxicity. BPA disrupts cellular redox balance by decreasing antioxidant defenses such as glutathione (GSH) and increasing lipid peroxidation markers, including malondialdehyde (MDA)[3,4]. However, the upstream regulatory mechanisms controlling antioxidant responses under BPA exposure remain insufficiently characterized.

The Kelch-like ECH-associated protein 1 (Keap-1)/Nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway is a principal endogenous antioxidant defense mechanism. Under oxidative stress conditions, Nrf-2 dissociates from Keap-1, translocates to the nucleus, and activates antioxidant response element-dependent gene expression[5]. Despite its established role in cellular defense, region-specific modulation of Keap-1/Nrf-2 signaling in the brain during environmentally relevant BPA exposure has not been comprehensively investigated in conjunction with behavioral outcomes.

Thymoquinone (TQ), the major bioactive component of Nigella sativa seed oil, is a lipophilic benzoquinone capable of crossing the blood-brain barrier. TQ exhibits antioxidant, anti-inflammatory, and anti-apoptotic properties, partly through activation of Nrf-2 signaling and enhancement of endogenous antioxidant capacity[5,6]. While previous studies have demonstrated the protective effects of TQ in various neurotoxicity models, an integrated methodological approach evaluating oxidative stress markers, Keap-1/Nrf-2 signaling, and behavioral parameters within the same BPA exposure paradigm is lacking[5,6,7].

Here, we describe a reproducible experimental protocol to assess oxidative stress biomarkers, Keap-1/Nrf-2 pathway expression, and behavioral alterations in a rat model of environmentally relevant BPA exposure, and to evaluate the neuroprotective effects of thymoquinone across multiple brain regions.

We hypothesized that low-dose BPA exposure induces behavioral impairments through oxidative stress-mediated dysregulation of the Keap-1/Nrf-2 signaling pathway, and that thymoquinone mitigates these effects by restoring redox balance and activating Nrf-2-dependent antioxidant responses.

Protocol

All experimental procedures were approved by the Institutional Animal Experimentation Local Ethics Committee (Approval No: 68429034/07).

Animals and experimental design

Twenty-four adult male Wistar Albino rats (200-250 g) were obtained from the Experimental Animal Research Laboratory and housed individually under controlled conditions (12 h light/dark cycle, 24 ± 2 °C) with *ad libitum* access to standard chow and tap water. Animals were acclimatized to laboratory conditions prior to the experiment, which lasted 32 days.

Rats were randomly assigned to experimental groups using a computer-generated simple randomization method to minimize selection bias, with equal numbers of animals allocated to each group (n = 6/group): control, BPA, BPA + 10 mg/kg thymoquinone (BPA + TQ I), and BPA + 20 mg/kg thymoquinone (BPA + TQ II). The sample size was determined by an a priori power analysis using G*Power software (version 3.1). Based on effect sizes reported in previous studies[5,6,7] evaluating BPA-induced neurotoxicity and antioxidant interventions, particularly for biochemical and behavioral outcome measures, a statistical power of 0.80 was assumed for one-way ANOVA. Under these conditions, a minimum of six animals per group was required to detect statistically significant differences among experimental groups.

BPA (50 µg/kg) and TQ (10 or 20 mg/kg) were administered intragastrically by gavage 1x daily for 30 days. All treatments were freshly prepared and administered at a total volume of 1 mL/kg. The BPA dose used in the present study (50 µg/kg/day) was selected based on its designation as a no-observed-adverse-effect level and reference dose by the United States Environmental Protection Agency and the U.S. Food and Drug Administration. This dose has been widely employed in experimental studies to model environmentally relevant BPA exposure and to investigate potential neurobehavioral and biochemical alterations occurring at exposure levels previously considered safe. Importantly, accumulating experimental evidence indicates that adverse neurological and behavioral effects may occur at or below this dose, supporting its relevance for assessing low-dose BPA toxicity[1,2]. The selection of TQ doses (10 and 20 mg/kg) was based on previous experimental studies demonstrating its neuroprotective and antioxidant efficacy without inducing toxicity[5,6,7]. These doses have been widely used in rodent models of neurotoxicity, oxidative stress, and neuroinflammation, and are well below reported toxicity thresholds for repeated administration. No pilot dose-finding study was conducted; dose selection was guided by literature precedence and established safety profiles to ensure both efficacy and translational relevance.

BPA was administered for 30 consecutive days. Behavioral assessments were conducted on days 31 and 32 following the final exposure. Animals were sacrificed on day 32 after completion of all behavioral tests. This timeline was designed to allow sufficient time for behavioral evaluation while minimizing potential confounding effects of acute handling or procedural stress on subsequent biochemical, molecular, and histopathological analyses. Thymoquinone was administered 1x daily immediately after BPA via the same intragastric route. Behavioral tests were performed following the final BPA and TQ administrations. The experimental timeline is summarized in **Figure 1**.

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Body weight measurement

Body weights were measured using a calibrated digital precision balance (±0.1 g sensitivity). Each rat was weighed individually in the morning before treatment administration, and measurements were recorded on days 1, 10, 20, and 32 under standardized conditions to minimize variability (**Figure 2**).

Behavioral assessments

All behavioral parameters were scored by a single observer blinded to group allocation to minimize observer bias and inter-observer variability. Animals were habituated to the testing room for at least 30 min prior to behavioral testing to reduce novelty-induced stress.

The combined use of the open field test (OFT) and forced swim test (FST) enables a comprehensive evaluation of BPA-induced neurobehavioral toxicity, as BPA has been shown to alter both emotional reactivity and locomotor activity in rodents[2,3]. OFT-derived locomotor outcomes were considered during interpretation of FST immobility to minimize potential confounding effects related to altered motor activity.

FST: Depression-like behavior was assessed using the forced swim test on day 32, preceded by a 15-min pre-test session conducted 24 h earlier. Rats were individually placed in cylindrical tanks (35 cm diameter, 50 cm height) filled with water (30 cm depth, 25 ± 1 °C) and allowed to swim for 5 min. Immobility, swimming, and climbing behaviors were recorded using a video camera and analyzed offline to evaluate behavioral despair[7].

OFT: Anxiety-like behavior and spontaneous locomotor activity were evaluated using the open field test on day 31. Each rat was placed individually in a white Plexiglas arena (90 cm x 35 cm) divided into 24 equal squares and allowed to explore freely for 5 min. Movements were recorded using a video camera. Time spent in central and peripheral zones and the number of crossings with all four paws were quantified as indices of anxiety-related behavior and locomotion[8].

Sample collection

On day 32, rats were deeply anesthetized with intramuscular ketamine (45 mg/kg) and xylazine (5 mg/kg). Adequate depth of anesthesia was confirmed by the absence of the pedal withdrawal reflex. Blood was collected via cardiac puncture using a sterile syringe, and approximately 3-5 mL of blood was obtained from each animal. Euthanasia was completed by exsanguination under deep anesthesia. Blood samples were transferred into anticoagulant-containing tubes and centrifuged at 3,000 x *g* for 10-15 min at 4 °C. The separated plasma was aliquoted and stored at −80 °C until biochemical analysis.

The hippocampus and left cerebral hemisphere were rapidly dissected, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent biochemical analyses, while the right cerebral hemisphere was fixed in 10% neutral buffered formalin for histopathological and immunohistochemical evaluations. To ensure methodological consistency and minimize inter-sample variability, the left hemisphere was systematically allocated for biochemical measurements and the right hemisphere for morphological analyses in all animals. Importantly, formalin fixation was strictly limited to tissues designated for histological and immunohistochemical procedures, and no fixed tissue was used for protein-based biochemical assays, thereby preventing fixation-related protein degradation. For biochemical assessments, defined amounts of frozen brain tissue (typically 50-100 mg per assay) were homogenized using assay-specific homogenization buffers and standardized tissue-to-buffer ratios according to the requirements of each biochemical parameter. All analyses were performed using equal tissue amounts within each assay across experimental groups.

Total protein concentration in tissue homogenates was determined using a fluorometric protein assay according to the manufacturer's instructions. All biochemical parameters were measured using standardized and equal protein concentrations within each assay to ensure comparability across samples and experimental groups.

Assessment of oxidative stress parameters

Lipid peroxidation was assessed by measuring MDA levels using the thiobarbituric acid reactive substances (TBARS) method in plasma and tissue samples[7]. Reduced thiol (RSH) and GSH levels were determined spectrophotometrically using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)-based assays[7]. Total nitric oxide (NO) levels in plasma and tissue homogenates were measured using the Griess reaction following nitrate reduction[7]. All biochemical parameters are expressed as nmol/g protein or µmol/g tissue, as indicated in the **Figure 3** and **Figure 4**. All biochemical assays were performed in duplicate, and mean values were used for statistical analysis.

In addition to brain tissue analyses, biochemical estimations were performed in blood samples to evaluate the systemic oxidative status induced by BPA exposure. Plasma lipid peroxidation levels were determined by the TBARS method, and results were expressed as MDA equivalents. Reduced sulfhydryl (RSH) levels were measured using DTNB, and total NO levels were assessed using the Griess reaction following nitrate reduction, as previously described[7]. Absorbance values were recorded spectrophotometrically at 532 nm (TBARS), 412 nm (RSH), and 540 nm (NO). All assays were performed in duplicate under standardized conditions.

BPA is a ubiquitous environmental toxicant that enters systemic circulation and exerts multi-organ effects; therefore, circulating oxidative stress markers provide complementary information reflecting overall redox imbalance[1,2]. Assessment of blood-based biochemical parameters allows evaluation of the relationship

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between central and peripheral oxidative alterations and enhances the translational relevance of the findings, as such markers are commonly used in environmental and clinical studies to monitor exposure-related toxicity.

Western blot analysis

Western blot analysis was performed to determine Keap-1 and Nrf-2 protein levels in hippocampal tissue. Approximately 50 mg of tissue was homogenized in 500 µL of RIPA buffer containing a protease inhibitor cocktail. Homogenates were centrifuged at 14,000 x *g* for 10 min at 4 °C, and the supernatants were collected. Protein concentrations were determined using a fluorometric protein quantification assay according to the manufacturer's instructions, and concentrations were calculated from a standard calibration curve. Equal amounts of protein (30 µg) were mixed with Laemmli buffer containing β-mercaptoethanol, heated at 95 °C for 5 min, separated on 10% SDS-PAGE gels, and transferred onto PVDF membranes at 110 V for 60-90 min. Membranes were blocked with 3% BSA and incubated overnight at 4 °C with primary antibodies against Keap-1 and Nrf-2 (1:3000) and β-actin (1:1000), followed by incubation with HRP-conjugated secondary antibodies. Protein bands were visualized by chemiluminescence, and band intensities were quantified by densitometric analysis and normalized to β-actin.

Histopathological analysis

Brain tissue samples from the right hemisphere were fixed in 10% neutral buffered formalin for 72 h, dehydrated through graded ethanol series, cleared in xylene, and embedded in paraffin according to standard histological procedures[9]. Fresh tissues were reserved for biochemical analyses. Coronal sections (5 µm) were obtained at the hippocampal and entorhinal cortex levels based on a rat brain atlas[10]. Four consecutive sections per animal were mounted on glass slides for histological and immunohistochemical evaluations, ensuring consistent section thickness and anatomical localization. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) following routine staining protocols[11]. Histopathological examination was performed under light microscopy by a single trained observer blinded to the experimental groups to minimize bias and eliminate inter-observer variability.

Immunohistochemical analysis

Regions of interest, including the hippocampus and entorhinal cortex, were selected based on their vulnerability to BPA-induced neurotoxicity and their roles in cognitive and emotional regulation[1,2]. Immunohistochemical staining for Nrf-2 and Keap-1 was performed on paraffin-embedded brain sections. Sections were deparaffinized in xylene, rehydrated through graded ethanol series, and subjected to heat-induced antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched prior to incubation.

Sections were incubated overnight at 4 °C with primary antibodies against Nrf-2 (1:100) and Keap-1 (1:200). Detection was performed using a commercially available, ready-to-use polymer-based HRP detection system containing species-specific secondary antibodies conjugated to horseradish peroxidase, according to the manufacturer's instructions. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) as the chromogen, followed by counterstaining with hematoxylin. Sections were dehydrated, cleared, and coverslipped.

Negative control sections were processed in parallel without primary antibody incubation. For quantitative analysis, three non-adjacent sections per animal were evaluated. Images were acquired under identical exposure settings for all groups. Sixteen non-overlapping fields per section were analyzed using ImageJ software. Regions of interest were manually delineated, background staining was subtracted, and immunoreactivity was expressed as mean gray value. The average value per animal was calculated and used for statistical analysis.

Histopathological scoring

Histopathological alterations were evaluated semi-quantitatively on H&E-stained sections. Ten randomly selected, non-overlapping fields per section were examined, and pathological parameters were scored independently. Damage severity in the hippocampus and entorhinal cortex was graded using a four-point scale based on the proportion of affected cells: score 0 (<5%), score 1 (≤ one-third), score 2 (one-third to two-thirds), and score 3 (> two-thirds). Mean scores per animal were used for statistical analysis.

Statistical analysis

Data were analyzed using SPSS software (version 29.0) and are presented as mean ± standard deviation (SD). Sample size was determined based on prior studies and power analysis, indicating that six animals per group provide >80% power to detect biologically relevant differences. Normality of data distribution was assessed using the Shapiro-Wilk test. Homogeneity of variances was evaluated using Levene's test. Group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

Results

Body weight

No statistically significant differences in body weight were observed between groups on days 1 and 32 of the study ($F(3,20)=2.750$, $p > 0.05$; **Figure 2**).

Behavioral test results

Open field test analysis revealed that BPA exposure significantly reduced locomotor activity, as indicated by a decreased number of crossings compared to the control group ($p < 0.001$). Treatment

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with 10 mg/kg and 20 mg/kg TQ significantly increased crossing numbers relative to the BPA group ($p < 0.001$), with a greater increase observed in the BPA+TQ II group compared to BPA+TQ I ($p < 0.001$; F(3,20)=183.031; **Table 1**).

BPA exposure significantly reduced the time spent in the center of the open field and increased time spent in the periphery compared to control ($p < 0.001$). Both TQ doses significantly reversed these effects, increasing center time and reducing periphery time ($p < 0.001$). The increase in center time was greater in the BPA+TQ II group than in the BPA+TQ I group ($p < 0.001$), whereas no significant difference was observed in periphery time between these groups ($p = 0.06$; F(3,20)=205.525 and F(3,20)=83.075, respectively; **Table 1**).

Forced swim test results demonstrated that BPA exposure significantly increased immobility time and decreased swimming and climbing durations compared to the control group ($p < 0.001$). Treatment with 10 mg/kg and 20 mg/kg TQ significantly reduced immobility time and increased swimming and climbing times relative to the BPA group ($p < 0.001$). The BPA+TQ II group exhibited a greater reduction in immobility time and a greater increase in swimming duration compared to BPA+TQ I ($p < 0.001$ and $p = 0.032$, respectively), while climbing time did not differ significantly between the two treatment groups ($p = 0.98$; F(3,20)=197.298, 692.778, and 738.646, respectively; **Table 2**).

Oxidative stress markers

In the cerebral cortex, BPA exposure significantly increased MDA and NO levels and decreased GSH levels compared to controls ($p < 0.001$). Co-treatment with 10 mg/kg and 20 mg/kg TQ significantly reduced MDA and NO levels and increased GSH levels compared to BPA alone ($p \leq 0.001$). Compared to BPA+TQ I, the BPA +TQ II group exhibited a greater reduction in MDA levels and a greater increase in GSH levels, whereas NO levels did not differ significantly between these groups ($p = 0.87$; F(3,20)=150.149, 55.556, and 100.143, respectively; **Figure 3 A-C**).

In plasma, BPA exposure significantly increased MDA and NO levels and decreased RSH levels compared to controls ($p < 0.001$). TQ treatment significantly reversed these alterations at both doses ($p < 0.001$). Compared to BPA+TQ I, the BPA+TQ II group showed a greater reduction in MDA levels and an increase in RSH levels, while NO levels were comparable between the two groups ($p=0.44$; F(3,20)=135.066, 57.67, and 100.213, respectively; **Figure 4 A-C**)

Keap-1 and Nrf-2 protein expression

BPA exposure significantly increased Keap-1 expression and decreased Nrf-2 levels compared to the control group ($p < 0.001$). TQ treatment significantly decreased Keap-1 expression and increased Nrf-2 levels compared to BPA alone, with more pronounced effects observed in the BPA+TQ II group ($p = 0.03$; F(3,20)=120.920 and 28.015, respectively; **Figure 5**, **Figure 6**, and **Figure 7**).

Histopathological and immunohistochemical findings

In H&E-stained brain sections, the control group exhibited normal histological architecture. In contrast, BPA exposure resulted in pronounced histopathological alterations, characterized by disrupted tissue organization, widespread neuronal loss, and necrotic areas within the parenchyma. Neurons showed evident degeneration, vacuolization, nuclear pyknosis, and partial cellular lysis. Increased inflammatory cell infiltration was observed, accompanied by marked edema and perivascular infiltration, indicating impaired vascular integrity. TQ treatment markedly attenuated these pathological alterations. In both BPA+TQ groups, neuronal morphology was partially preserved, cell density increased, and inflammatory infiltration was reduced. Nevertheless, mild cellular disorganization and congestion persisted in the BPA+TQ I group. In contrast, the BPA+TQ II group demonstrated a substantial improvement in histological architecture, with tissue morphology closely resembling that of the control group (**Figure 8**).

Immunohistochemical analysis revealed that Nrf-2 immunoreactivity was predominantly localized in the cytoplasm in the control group. In the BPA-exposed group, Nrf-2 staining intensity was markedly reduced compared to the control. In contrast, TQ administration significantly increased Nrf-2 immunoreactivity in BPA-treated rats, with a more pronounced effect observed in the BPA+TQ II group (**Figure 9**).

Keap-1 expression showed weak cytoplasmic staining in neuronal cells of the control group. BPA exposure resulted in a marked increase in Keap-1 immunoreactivity, characterized by strong cytoplasmic and nuclear staining. Treatment with TQ significantly reduced Keap-1 expression compared to the BPA group, with the BPA+TQ II group displaying staining intensity closest to control levels (**Figure 9**).

Quantitative analysis confirmed a significant decrease in Nrf-2 immunoreactivity in the BPA group compared to controls ($p < 0.001$), whereas both BPA+TQ treatment groups exhibited significantly increased Nrf-2 staining relative to BPA alone ($p < 0.01$). Keap-1 immunoreactivity was significantly elevated following BPA exposure ($p < 0.001$) and was markedly reduced by TQ treatment in the BPA+TQ I ($p < 0.01$) and BPA+TQ II ($p < 0.001$) groups, approaching control values (**Figure 10**).

Semi-quantitative histopathological scoring of brain tissue

Semi-quantitative histopathological evaluation revealed marked brain tissue injury in the BPA group. Rats exposed to BPA showed significantly higher scores for neuronal loss, cellular edema with nuclear pyknosis, vascular congestion, and inflammatory cell infiltration compared with the sham group ($p < 0.05$). In contrast, no significant histopathological alterations were observed in the BPA+TQ I or BPA+TQ II groups compared with the control group ($p > 0.05$). Both thymoquinone-treated groups exhibited significantly

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lower scores across all evaluated parameters relative to the BPA group ($p < 0.05$). Median histopathological scores in the BPA+TQ I and BPA+TQ II groups were comparable to those of the control group, indicating a substantial attenuation of BPA-induced brain tissue damage (**Table 3**).

Overall, BPA exposure resulted in pronounced oxidative stress, disruption of Keap-1/Nrf-2 signaling, behavioral impairments, and histopathological damage in rat brain tissue compared with the control group. These alterations were consistently observed across biochemical, molecular, behavioral, and histological assessments in the BPA-treated rats. Treatment with TQ significantly attenuated these BPA-induced effects in a dose-dependent manner. Both 10 mg/kg and 20 mg/kg TQ reduced oxidative stress markers, restored antioxidant capacity, normalized Keap-1 and Nrf-2 expression levels, improved anxiety- and depression-like behaviors, and alleviated histopathological damage, with values approaching those observed in the control group. The inclusion of control, BPA-only, and BPA+TQ treatment groups enabled clear differentiation between toxic effects and treatment-related improvements. Notably, the protective effects were more pronounced in the 20 mg/kg TQ-treated group, which consistently showed greater biochemical, molecular, behavioral, and histological improvement compared with the lower dose.

Data availability:

All data are included within the article.

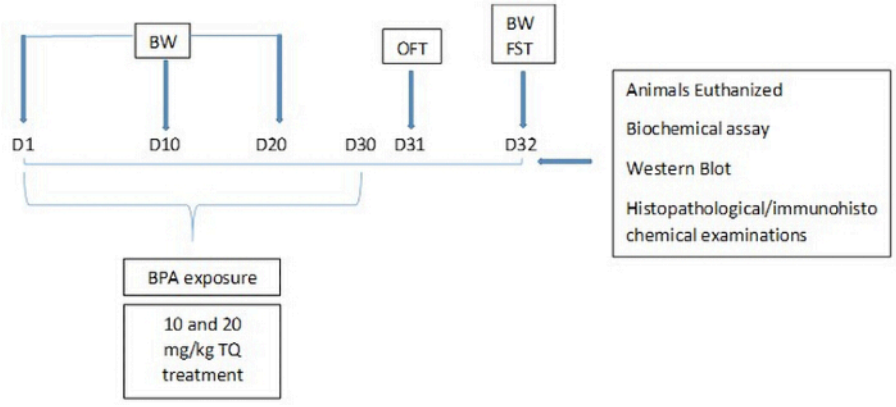

Figure 1: Experimental protocol. Timeline illustrating the sequence of experimental procedures. D indicates the experimental day. Rats were subjected to the open field test (OFT) on day 31 and the forced swim test (FST) on day 32. Body weight (BW) was recorded on days 1, 10, 20, and 32. Please click here to view a larger version of this figure.

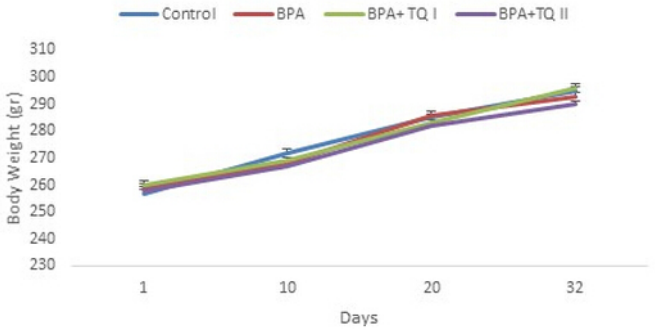

Figure 2: Body weight measurements. Body weight was recorded on day 1 and day 32 of the experimental period. Values are presented as mean ± SD (n=6). No significant differences were observed between experimental groups. Please click here to view a larger version of this figure.

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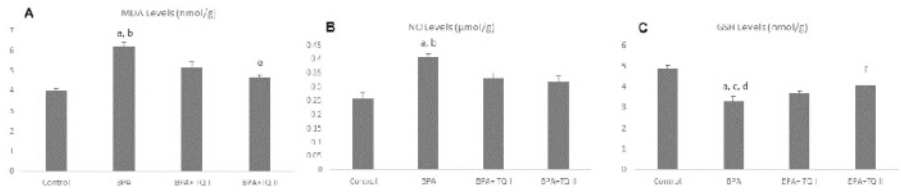

Figure 3: Oxidative stress markers in the cerebral cortex. (A) Malondialdehyde (MDA), **(B)** Nitric oxide (NO), and **(C)** Glutathione (GSH) levels were measured in cerebral cortex tissue. Values are presented as mean ± SD (n=6). Different superscript letters indicate statistically significant differences between groups for the same parameter (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Please click here to view a larger version of this figure.

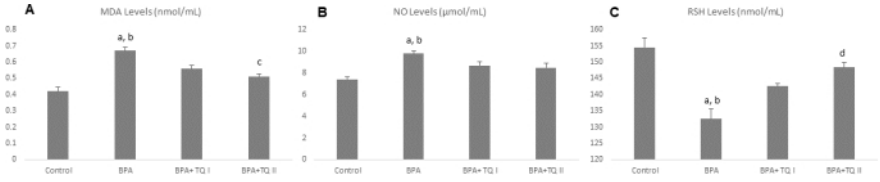

Figure 4: Plasma oxidative stress parameters. (A) Malondialdehyde (MDA), **(B)** Nitric oxide (NO), and **(C)** Reduced sulfhydryl groups (RSH) levels were measured in plasma. Values are presented as mean ± SD (n=6). Different superscript letters indicate statistically significant differences between groups for the same parameter (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Please click here to view a larger version of this figure.

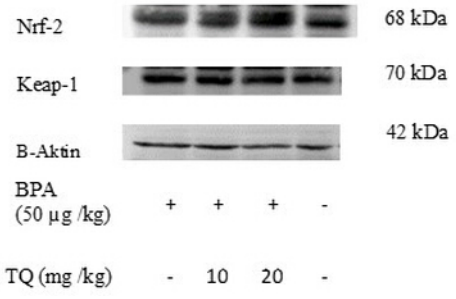

Figure 5: Western blot analysis of Keap-1 and Nrf-2. Representative Western blot images and densitometric analysis of Keap-1 and Nrf-2 protein expression in hippocampal tissue. Protein levels were normalized to the housekeeping protein and expressed relative to the control group. Please click here to view a larger version of this figure.

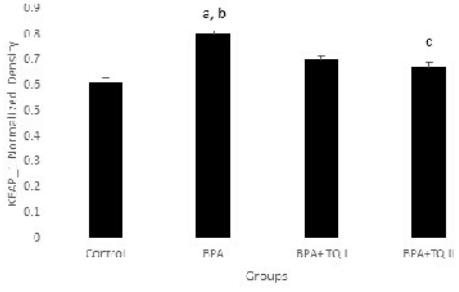

Figure 6: Keap-1 protein expression in the hippocampus. Normalized Keap-1 protein expression in hippocampal tissue. Values are presented as mean ± SD (n=6). Different superscript letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Please click here to view a larger version of this figure.

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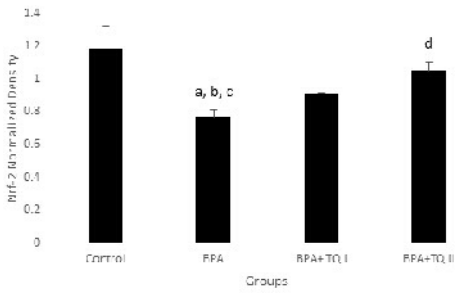

Figure 7: Nrf-2 protein expression in the hippocampus. Normalized Nrf-2 protein expression in hippocampal tissue. Values are presented as mean ± SD (n=6). Different superscript letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Please click here to view a larger version of this figure.

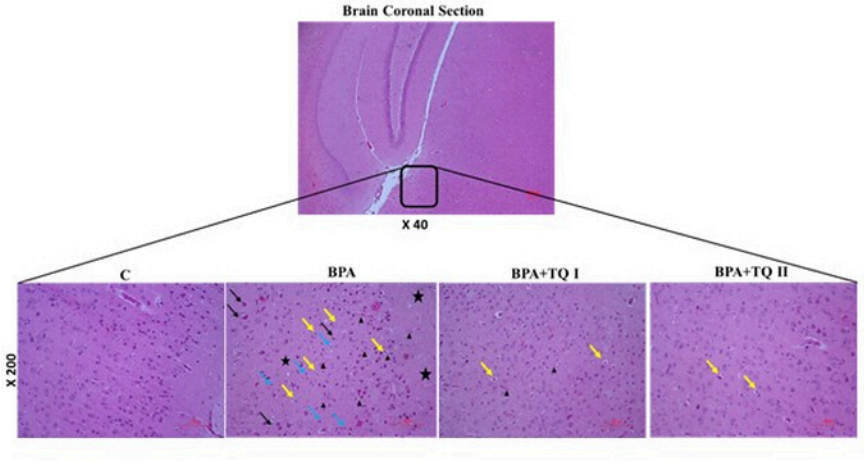

Figure 8: Histopathological evaluation of brain tissue. Representative hematoxylin and eosin-stained coronal brain sections at the hippocampal and entorhinal cortex levels; scale bar=100 µm. The control group shows normal neuronal and tissue architecture. The BPA group exhibits neuronal degeneration, edema, nuclear pyknosis, inflammatory infiltration, and vascular congestion. In the BPA + TQ I and BPA + TQ II groups, thymoquinone treatment partially preserved tissue integrity and neuronal morphology. Please click here to view a larger version of this figure.

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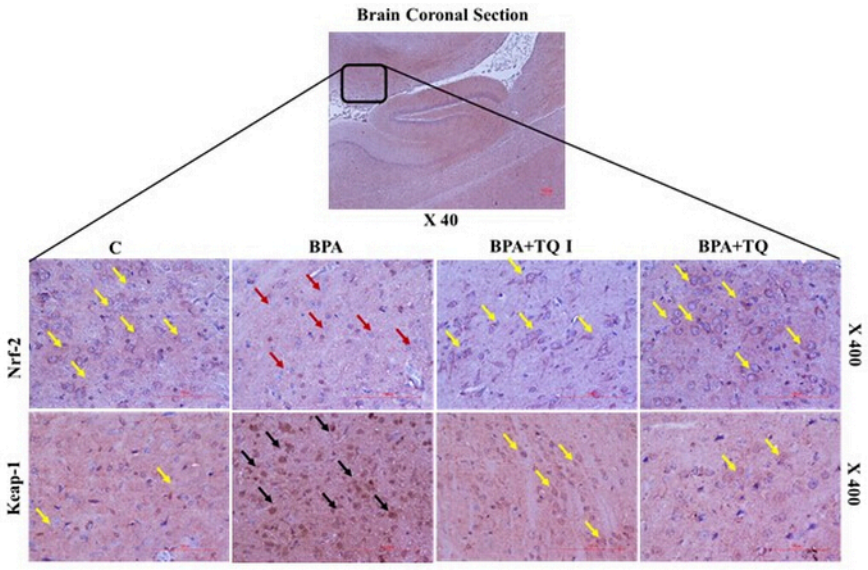

Figure 9: Immunohistochemical localization of Nrf-2 and Keap-1. Immunohistochemical staining of Nrf-2 and Keap-1 in coronal brain sections at the hippocampal and entorhinal cortex levels from Control, BPA, BPA + TQ I, and BPA + TQ II groups. Scale bar=100 µm; original magnification 400x. Yellow arrows indicate cytoplasmic staining, red arrows indicate reduced cytoplasmic staining, and black arrows indicate combined nuclear and cytoplasmic staining. Please click here to view a larger version of this figure.

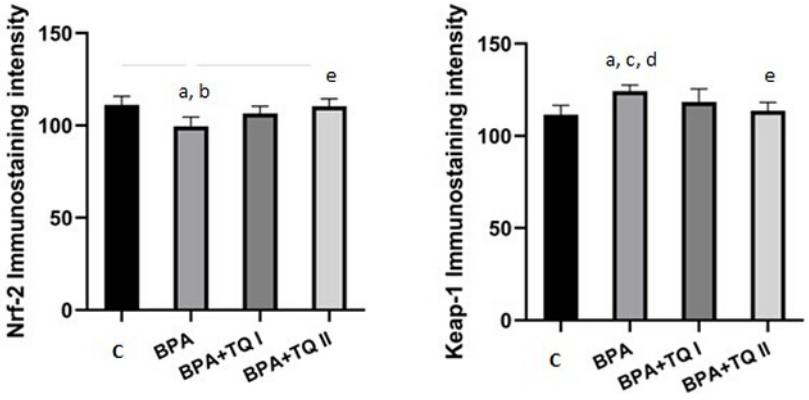

Figure 10: Quantitative analysis of Nrf-2 and Keap-1 immunoreactivity. Semi-quantitative comparison of Nrf-2 and Keap-1 expression in the hippocampal and entorhinal cortex regions. Values are presented as mean ± SD (n=6). Different superscript letters indicate statistically significant differences between groups (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Please click here to view a larger version of this figure.

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Group	Immobility Time (s)	Climbing Time (s)	Swimming Time (s)
Control	50 ± 4	98 ± 6	145 ± 5
BPA	145 ± 6 [a, b]	72 ± 7 [a, d, e]	88 ± 5 [a, b]
BPA + TQ I	105 ± 5	85 ± 5 [f]	104 ± 4 [g]
BPA + TQ II	90 ± 4 [c]	88 ± 5 [f]	112 ± 5 [g]

Table 1: Effects of BPA and thymoquinone on forced swim test performance. The forced swim test was used to assess depression-like behavior. Immobility, climbing, and swimming times were recorded during the test session. Data are presented as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Time is expressed in s. Different superscript letters indicate statistically significant differences between groups ($p < 0.05$).

Group	Number of Crossings	Time Spent in the Center (s)	Time Spent in the Periphery (s)
Control	90 ± 3	168 ± 4	130 ± 5
BPA	55 ± 4 [a, b]	105 ± 8 [a, b]	200 ± 10 [a, b]
BPA + TQ I	68 ± 3	132 ± 5	165 ± 6 [d]
BPA + TQ II	80 ± 3 [c]	150 ± 4 [c]	150 ± 5 [d]

Table 2: Effects of BPA and thymoquinone on open field test parameters. The open field test was used to evaluate locomotor activity and anxiety-like behavior. The number of crossings, time spent in the center, and time spent in the periphery were recorded. Data are presented as mean ± SD (n=6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Time is expressed in s. Different superscript letters indicate statistically significant differences between groups ($p < 0.05$).

Parameter	Control	BPA	BPA + TQ I	BPA + TQ II
Neuronal Loss	0.00 [0.0–1.0] [a]	2.00 [2.0–3.0] [c]	0.00 [0.0–1.0] [a]	0.00 [0.0–1.0] [a]
Cellular Edema& Nuclear Pyknosis	0.00 [0.0–1.0] [a]	2.00 [2.0–3.0] [c]	0.00 [0.0–1.0] [a]	0.00 [0.0–1.0] [a]
Vascular Congestion	0.00 [0.0–1.0] [a]	3.00 [2.0–3.0] [c]	0.00 [0.0–1.0] [a]	0.00 [0.0–1.0] [a]
Inflammatory infiltration	0.00 [0.0–1.0] [a]	2.00 [2.0–3.0] [c]	0.00 [0.0–1.0] [a]	0.00 [0.0–1.0] [a]

Table 3: Semi-quantitative histopathological scoring of brain tissue. Histopathological alterations were evaluated using a semi-quantitative scoring system ranging from 0 to 3. Data are presented as median [minimum-maximum]. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. Different superscript letters indicate statistically significant differences between groups for the same parameter ($p < 0.05$).

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Discussion

The present study evaluated the effects of BPA, a structural component of polycarbonate plastics considered safe by the USEPA at a dose of 50 µg/kg[1,2], on oxidative stress, Keap-1/Nrf-2 signaling, behavior, and histopathological alterations in the rat brain. In addition, the potential neuroprotective effects of TQ, a naturally occurring compound with well-documented antioxidant properties, were investigated at two different doses (10 mg/kg and 20 mg/kg)[5,6,7]. The findings demonstrate that subchronic exposure to a safe dose of BPA induces significant oxidative imbalance, disrupts Keap-1/Nrf-2 signaling, impairs behavior, and causes marked histopathological damage, while concurrent TQ treatment effectively mitigates these alterations in a dose-dependent manner.

In this study, BPA exposure for 30 days resulted in increased Keap-1 expression and decreased Nrf-2 levels in the hippocampus, accompanied by elevated MDA and NO levels and reduced GSH/RSH concentrations in the cerebral cortex and plasma. Recent studies have similarly demonstrated that environmentally relevant BPA exposure induces oxidative stress-mediated neurotoxicity and behavioral impairments through suppression of antioxidant signaling pathways, including Nrf-2, in hippocampal and cortical regions[3,12,13,14,15,16,17].

Differences observed between our findings and some studies reporting decreased Keap-1 expression following BPA exposure[18,19] may be attributed to variations in BPA dose, exposure duration, and tissue specificity, particularly under high-dose and long-term exposure conditions in testicular tissue. Importantly, co-administration of TQ at both 10 mg/kg and 20 mg/kg significantly reduced Keap-1 levels while restoring Nrf-2 expression in the hippocampus, supporting the hypothesis that TQ exerts its antioxidant effects, at least in part, through modulation of the Keap-1/Nrf-2 signaling pathway. Consistent with the molecular findings, TQ treatment markedly reduced oxidative stress markers and restored endogenous antioxidant capacity in BPA-exposed rats. The most pronounced improvements were observed in the BPA+TQ II group (20 mg/kg), where biochemical parameters approached control values. These findings are in line with previous reports demonstrating that thymoquinone and related antioxidant compounds attenuate toxin-induced oxidative damage, improve behavioral outcomes, and preserve neuronal integrity in experimental neurotoxicity models[5,6,13,14,20,21,22,23]. The parallel alterations observed in both brain tissue and plasma oxidative stress markers suggest that BPA-induced neurotoxicity is accompanied by systemic redox imbalance, supporting the relevance of peripheral biomarkers in reflecting central nervous system oxidative status.

Body weight analysis revealed that 30 days of BPA exposure at 50 µg/kg did not result in significant changes, consistent with several studies using similar doses and exposure durations[1,2]. Discrepancies with reports showing increased or decreased body weight following BPA exposure may be related to differences in administration route, developmental exposure windows, and BPA dose[24,25,26]. Similarly, TQ treatment at both doses did not alter body weight, supporting its tolerability and consistency with earlier findings[6,20].

Behavioral assessments demonstrated that BPA exposure significantly reduced locomotor activity, increased anxiety-like behavior in the open field test, and induced depression-like behavior in the forced swim test. These findings are in agreement with extensive literature indicating that BPA adversely affects emotional and motor behaviors across different exposure paradigms[1,2,3,12,25,26,27]. Treatment with TQ at both 10 mg/kg and 20 mg/kg significantly improved locomotor activity, reduced anxiety-like behavior, and alleviated depression-like responses, with the most robust behavioral recovery observed in the BPA +TQ II group. Although direct studies examining TQ effects on behavior following BPA exposure are limited, related studies using BPA derivatives and other neurotoxicity models support the antidepressant and anxiolytic potential of TQ[7,20,22]. These behavioral improvements should therefore be interpreted as attenuation of BPA-associated disturbances rather than direct psychotropic effects of thymoquinone. Importantly, interpretation of forced swim test immobility was performed in conjunction with open field test locomotor outcomes to minimize potential confounding effects related to altered motor activity.

Histopathological analysis further corroborated the biochemical and behavioral findings. BPA exposure caused severe disruption of cerebral cortex architecture, neuronal loss, necrosis, and inflammatory cell infiltration, consistent with previous reports of BPA-induced neurodegeneration[3,16,20]. Co-treatment with TQ significantly preserved neuronal morphology, reduced inflammatory infiltration, and improved tissue organization, particularly at the higher dose. These histological improvements support the notion that TQ-mediated activation of antioxidant defenses contributes to structural neuroprotection.

This study advances the field by providing an integrated experimental framework combining behavioral testing, oxidative stress assessment, molecular signaling analysis, and histopathological evaluation to assess BPA-induced neurotoxicity and therapeutic intervention. To our knowledge, this study provides one of the first integrated demonstrations that thymoquinone modulates the Keap-1/Nrf-2 signaling pathway in the rat brain under subchronic, environmentally relevant BPA exposure.

Several limitations of the present study should be acknowledged. First, the experiments were conducted exclusively in male

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rats; therefore, potential sex-specific differences in BPA-induced neurotoxicity and thymoquinone responsiveness were not evaluated. Second, a thymoquinone-only treatment group (10 mg/kg or 20 mg/kg in the absence of BPA) was not included. Consequently, the intrinsic neurobehavioral and antioxidant effects of thymoquinone under physiological conditions could not be directly assessed, and the observed findings should be interpreted as protective associations rather than definitive pharmacological actions of thymoquinone. This experimental design was intentionally selected to minimize animal use in accordance with ethical principles and to focus primarily on the protective potential of thymoquinone against BPA-induced neurotoxicity.

In addition, although the BPA dose (50 µg/kg/day) was selected based on established reference doses and is widely used to model environmentally relevant low-dose exposure, the internal BPA burden was not quantified in plasma or brain tissue. The absence of toxicokinetic measurements limits the precise interpretation of tissue-level exposure following chronic gavage administration. Moreover, while alterations in Nrf-2 and Keap-1 protein expression were evaluated, downstream antioxidant target genes and Nrf-2 nuclear translocation were not assessed, restricting mechanistic conclusions regarding pathway activation.

Finally, although TBARS is a commonly used indicator of lipid peroxidation, it lacks complete specificity for malondialdehyde[7]. To mitigate this limitation, TBARS findings were interpreted in conjunction with complementary oxidative stress parameters, including reduced thiol (GSH/RSH) and nitric oxide levels. Future studies incorporating sex-based comparisons, thymoquinone-only control groups, toxicokinetic assessments, and comprehensive molecular analyses of Nrf-2 downstream signaling are warranted to further elucidate the mechanisms underlying BPA-induced neurotoxicity and the modulatory effects of thymoquinone.

In conclusion, the present findings demonstrate that BPA, even at a dose considered safe, induces significant neurotoxic effects through oxidative stress and disruption of Keap-1/Nrf-2 signaling in rat brain tissue. Thymoquinone treatment, particularly at 20 mg/kg, effectively counteracted these effects by restoring antioxidant defenses, improving behavioral outcomes, and preserving brain histoarchitecture. These results indicate that thymoquinone may represent a promising neuroprotective agent against environmental toxin-induced neurotoxicity. Future studies should focus on elucidating downstream Nrf-2-regulated antioxidant and mitochondrial pathways, evaluating the long-term efficacy of thymoquinone in chronic BPA exposure models, and determining whether similar neuroprotective effects are observed across different sexes and developmental stages. Additionally, translational studies are warranted to further explore the therapeutic potential of thymoquinone against environmental neurotoxicants.

Disclosures

The authors have no conflicts of interest to declare.

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