Effect of thymoquinone on the structure of the cerebral cortex of adult male albino rats treated with tramadol

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Background Pain-associated depression is a symptom of many diseases such as cancer, and postoperative and myocardial infarction. Tramadol (TRM) is a centrally acting synthetic opioid, similar to an analgesic, used worldwide to treat severe pain with an anti-depressant-like effect. TRM is more popular abused among adults in most countries to relive pain and increase sexual activities. Thymoquinone (TQ), a volatile oil, is one of the main constituents of *Nigella sativa* seeds. It has anti-inflammatory, antioxidant, anticonvulsant, antitussive, and anti-tumor effects.

The aim of work The present study was designed to evaluate the effects of TRM on the structure of cerebral cortex of the adult male albino rats and the possible impact of using TQ to improve these changes and to test the analgesic, anti-depressant, and antioxidant effects of TRM and/or TQ.

Materials and methods Forty-eight male albino rats weighting 180–200 g were used in the present study. The rats were divided into four groups: control group (GI): 12 rats received food and water. TQ group (GII): 12 rats received an oral dose of TQ (20 mg/kg) for 4 weeks. TRM group (GIII): 12 rats received an oral dose of TRM HCl (50 mg/kg) for 4 weeks. Combined group (GIV): 12 rats received both TRM (50 mg/kg) and TQ (20 mg/kg) for 4 weeks.

Results TQ supplementation significantly increased the analgesic effect of TRM after acute and chronic treatment by the thermal and chemical methods and attenuated the development of tolerance. TQ also significantly improved the anti-depressant effect of TRM. Furthermore, TQ significantly increased the suppressed levels of glutathione content and activities of superoxide dismutase, catalase, and glutathione peroxidase induced by TRM. It also significantly reduced the elevated levels of malondialdehyde and nitric oxide caused by TRM. Histological examination of TRM-treated cerebral cortex showed distortion of its layers, increased vascularity, and cellularity, with a significantly increased number of apoptotic cells. TRM also induced a significant increase in the mean area percentage of both apoptotic index and the optical density of BAX immune-stain compared with the control group. These changes were improved in TQ-treated rats.

Conclusion TQ supplementation improved the analgesic, anti-depressant effects of TRM, with an improvement in the cerebral cortex structure and antioxidant markers and amelioration of oxidative stress markers. Furthermore, it attenuated TRM tolerance and neurotoxic effects.

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Introduction

Tramadol (TRM) is a synthetic centrally acting opioid-like analgesic that was firstly registered in 1998. It is available in oral and parenteral dosage forms for the treatment of pain syndromes [1]. TRM was originally considered to have a much better safety profile than other opioid analgesics [2].

The analgesic and anti-depressant efficacy of TRM can be attributed to its partial affinity for the μ-opioid receptors and its inhibition of norepinephrine and serotonin reuptake [3]. It was reported that increasing serotonin and norepinephrine levels reduced the inflammatory cytokines that are released by the brain in response to stress, which explains the anti-inflammatory effect of TRM [4,5].

TRM can cause psychological and physical addiction similar to that of other opiates and, in some cases, may lead to lethal health effects [6,7]. TRM abuse became more popular among teens in most countries. Teen ager abuse TRM to increase their work effectiveness and their sexual activities. So they addicts TRM as substitution for heroin, which might lead to the accumulation of toxic metabolites in the body [8,9], which carries all possible opiates risks, including dizziness, headache, somnolence, nausea, constipation, sweating, pruritus, and central nervous system stimulation [10]. TRM causes respiratory depression [11] associated with the development of a physical dependence and a severe withdrawal syndrome [12,13].

Thymoquinone (TQ) (2-methyl-5-isopropyl-1, 4-benzoquinone) is a bioactive monomer [14] and it

is the main constituent (about 30–48%) of black seed (*Nigella sativa*) oil [15]. *N. sativa* is a plant used extensively in traditional medicine in Arabian countries for the treatment of many pathological conditions such as hypertension, dysentery, asthma, and eczema [16]. TQ has different properties in modern pharmacology as it contains an active quinone that exerts many therapeutic effects through analgesic and anti-inflammatory activities [17].

It has been shown that TQ is a potent mitochondria-targeted antioxidant that is effective in treating a large number of age-related pathologies [18]. TQ protects the organs against oxidative damage induced by a variety of free radical-generating conditions and chemotherapeutic agents, and allergic encephalomyelitis [19]. TQ also exerts anti-apoptotic [15], anticonvulsant, antitussive, analgesic [20], and anti-tumor effects [21]. TQ exerts immune-modulatory [22], antihistaminic, and antidiabetic effects [23]. TQ also has hepatoprotective activities [24]. Various mechanisms of action of TQ have been reported as antioxidant or pro-oxidant at different concentrations [25].

The cerebrum is the principal part of the brain that contains the cell bodies of nerves, that integrate and coordinate the activity of other nerves, as well as the nerves and neural pathways that constitute memory. The chronic effect of using TRM drug on cerebral neurons suggests that long-term use of opioids may induce structural alterations in neurons [26].

Aim of the work

The present study was designed to study the neurotoxic effects of TRM on the structure of the cerebral cortex of adult male albino rats and the possible impact of using TQ to induce improvements in these changes. This study also aimed to measure the analgesic, anti-depressant, and antioxidant effects of TRM and TQ.

Materials and methods

Drugs

(1) TRM hydrochloride (Tamol-X tablets, 225 mg) manufactured by Royal Pharmaceutical Industries (Cairo, Egypt), was used in the present study. The doses for rats were calculated according to Paget and Barnes [27]. The dose for adult rats ranged from 45 to 90 mg/kg bw/day, which corresponds to an adult human therapeutic dose [28]. The drug was dissolved in distilled water and administered at a dose of 50 mg/kg by an oral gavage once daily for 4 weeks.

(2) TQ was purchased from Sigma Aldrich Corp. (St Louis, Missouri, USA). It was used in the form of a yellowish crystalline powder soluble in water. It was freshly prepared by dissolving in distilled water and administered at a dose of 20 mg/kg by an oral gavage once daily for 4 weeks [29].

Animals

Forty-eight adult male albino rats of the local strain weighing 180–200 g were used in the present study. Rats were housed in stainless-steel mashed cages 60×30×30 cm (five rats/cage). Rats were kept for 15 days for laboratory acclimatization before starting the experiment. Rats were kept under strict care and hygienic conditions at room temperature and were fed on the rat chow diet and allowed free access to water in the animal house at the Faculty of Medicine for Girls, Al-Azhar University.

Experimental design

Animals were divided into four groups:

(1) The control group (GI): 12 rats divided into two subgroups
   (a) GI a: six rats were administered food and water daily for 4 weeks.
   (b) GI b: six rats were exposed to chemical pain by injecting acetic acid (10 mg/kg intraperitoneally) for the Writhing test.
(2) TQ group (GII): 12 rats were divided into two subgroups:
   (a) GII a: six rats received TQ (20 mg/kg orally) by an oral gavage once daily for 4 weeks.
   (b) GII b: six rats were exposed to chemical pain by injecting acetic acid (10 mg/kg intraperitoneally) for the Writhing test in addition to TQ (20 mg/kg orally).
(3) TRM group (GIII): 12 rats were divided into two subgroups:
   (a) GIII a: six rats received TRM HCl (50 mg/kg orally) by an oral gavage once daily for 4 weeks.
   (b) GIII b: six rats were exposed to chemical pain by injecting acetic acid (10 mg/kg intraperitoneally) for the Writhing test in addition to TRM HCl (50 mg/kg orally).
(4) Combined (TRM+TQ group) (GIV): 12 rats were divided into two subgroups:
   (a) GIV a: six rats received both TRM HCl and TQ (50 mg/kg) and (20 mg/kg/day), respectively, orally for 4 weeks.
   (b) GIV b: six rats were exposed to chemical pain by injecting acetic acid (10 mg/kg intraperitoneally) for the Writhing test in addition to TQ (20 mg/kg orally) and TRM HCl (50 mg/kg orally).

Methods

Pharmacological study

(1) Analgesic effect: induction of pain and evaluation of analgesic effects were performed using two methods:
   (a) Thermal method: hotplate test [30].
   The hotplate test was carried out in all groups after acute (day 1) and chronic treatment (day 28) in (GI a, GII a, GIII a, and GIV a). Each rat was placed on the hotplate at 55°C.
   (b) Chemical method: Writhings test [31].
   The analgesic effect was tested in the (GI b, GII b, GIII b, and GIV b) groups by injecting acetic acid (10 mg/kg intraperitoneally) on day 1 for the Writhing test; the number of contractions (Writhing) in each rat was counted for the next 15 min. The inhibition % was calculated using the following formula: Inhibition present=(1–WT/WC)×100.
   Where WT and WC represent the number of Writhing's in the treated and control groups, respectively. All animals in this method died within 30–45 min.

(2) Anti-depressant test: forced-swim test [32].

All rats (GI a, GII a, GIII a, and GIV a) (in day 27) were forced to swim in a cylinder containing fresh water (T 22±2°) for 15 min. This was the pretest swim; 24 h later, each rat was re-exposed to the forced-swim test in a similar environment for 5 min. ' Test session,' the total duration of swimming, climbing, and immobility time/s were recorded for each animal in different groups.

Biochemical assay

This was used to determine the effect of TRM and or TQ on reduced glutathione (GSH) content, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-px) activities and on malondialdehyde (MDA) and nitric oxide (NO) levels.

Samples collection

At the end of the experiment, the whole blood of each rat was collected by heart puncture under light ether anesthesia in a heparinized tube. Each sample was divided into two heparinized tubes. One tube was used for the determination of the GSH content, SOD, CAT, and GSH-px activities, whereas the other tube was centrifuged and the separated plasma was used for the determination of MDA and NO.

Determination of GSH was performed according to Beutler *et al.* [33], SOD according to Minami and Yoshikawa [34], CAT according to Bergmeyer [35], and GSH-px activity according to the method of Gross *et al.* and Necheles *et al.* [36,37], whereas lipid peroxidation was determined by measuring the MDA level according to the method of Yoshioka *et al.* [38] and NO was evaluated using the method described by Miranda *et al.* [39].

Histological methods

Light microscopic examination

At the end of the experiment, the animals were anesthetized with ether inhalation. Then, intracardiac perfusion was performed by 2% glutaraldehyde for partial fixation of the brain. The brains were dissected and then the left frontal cortex was obtained and prepared for the histological examination. Specimens were fixed in 10% neutral-buffered formalin for 48 h for light microscopic examination, dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin. Sections were cut at 5 μm using a microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E) for routine histological examination [40]. Cresyl violet stain was used to distinguish between the viable normal cells and degenerated neurons [41].

Immunohistochemical study

The immunohistochemical staining was used to test the protein expression of BAX in hepatocytes of different groups. BAX is a pro-apoptotic protein that promotes apoptosis [42]. Cerebral cortex samples were fixed in 10% formalin, dehydrated in ascending grades of alcohol, cleared, embedded in paraffin blocks, and were cut into sections of 5 μm thickness. Sections were obtained on positive slides and immune-stained using an avidin–biotin technique. The protein expression level was evaluated using a standard three-step immunohistochemical procedure. BAX antibodies were used as the primary antibody, and then the biotinylated (contained biotin) secondary antibody was added, followed by horseradish peroxidase conjugated with streptavidin. As streptavidin has a high affinity to biotin, it bind to the place where the primary antibody coated the background, thus after adding a chromatogen as 3, 3-diaminobenzidine, a brown color appears. Counter staining was performed by hematoxylin, where liver tissue appeared blue. For each preparation, a negative control was performed (a slide without the primary antibody) by replacing the primary antibody with PBS [43]. Immunohistochemistry was performed at The Cancer Institute, Cairo University.

Then, a morphometric study was carried out to count the apoptotic cells to calculate the cerebral apoptotic index (AI) and measure the optical density of the BAX reaction.

The histomorphometrical analysis

This was done using Lica Qwin 500 LTD image analysis; the AI was determined by counting the total number of at least 1000 cells per slide in 10 fields chosen randomly from all layers of the cerebral cortex at 200 magnification. AI=(number of immune-positive cerebral cortex cells/total number of cerebral cortex cells)×100 [44].

Statistical analysis

The recorded data were presented as mean±SE. One-way analysis of variance was carried out and the statistical comparisons among the groups were performed with Duncan's test using a statistical package program (SPSS, version 17.0). *P* value less than or equal to 0.05 was considered significant for all statistical analyses.

Results

Pharmacological study

Analgesic effect

(1) Thermal method (hotplate test):

(a) Effect of TQ and/or TRM on reactive time (s) in response to hotplate stimulus day 1 (acute treatment) (GI a, GII a, GIII a, and GIV a).

An oral treatment for day 1 (acute) of TQ (20 mg/kg orally) and/or TRH (50 mg/kg orally) suppressed the nociceptive response in the hotplate test as the mean reactiontime was significantly increased in the different groups compared with the control group. Comparison studies between different groups were found to be statistically significant at *P* value less than 0.001 (Table 1 and Fig. 1).

(b) Effect of TQ and/or TRM on the reaction time (s) in response to hotplate stimulus day 28 (chronic treatment) (GI a, GII a, GIII a, and GIV a).

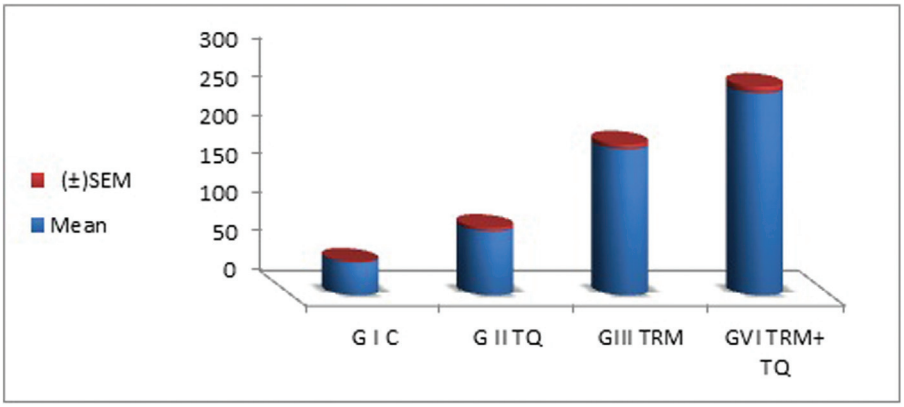
An oral treatment daily (28 days) of TQ (20 mg/kg orally) and/or TRM (50 mg/kg orally) suppressed the nociceptive response to the hotplate stimulus. The mean reaction time increased significantly in all treated groups versus the control group, comparison studies between different groups were found to be statistically significant at *P* value less than 0.001. However, an insignificant difference was observed in the comparison between the TRM-treated group on day 1 (acute treatment) and the TRM-treated group on day 28 (chronic treatment), *P* value more than 0.005 (Table 2 and Fig. 2).

Table 1 Mean % changes in the reaction time (s) to a hotplate stimulus of thymoquinone (20 mg/kg orally) and/ or tramadol (50 mg/kg orally) day 1 (acute treatment)

Number (*N*=6)	Mean±SEM	*P* value
Control (GIa)	42±1.2	$P<0.001$
TQ treated (GIIa)	82.41±4.5	$P^{a}<0.001$
TRM treated (GIIIa)	188.33±6.1	$P^{a+b}<0.001$
TRM+TQ treated (GIVa)	260.6±8.3	$P^{c}<0.001$

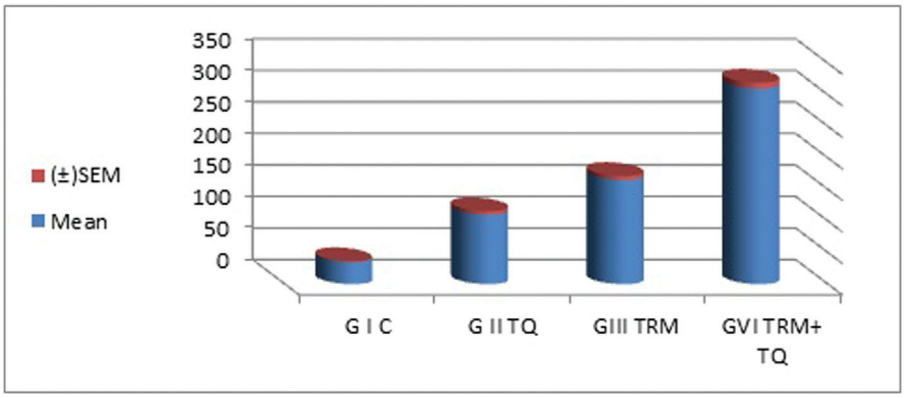
TQ, thymoquinone; TRM, tramadol. P^{a}, significant difference between all-treated groups versus the control group, *P* value less than 0.001. P^{b}, significant difference between TRM-treated groups versus the TQ group, *P* value less than 0.001 P^{c}, significant difference between combined TRM+TQ-treated groups versus either the TRM-treated and/or the TQ-treated group.

Figure 1



Mean % changes in the reaction time (s) to hotplate stimulus TQ (20 mg/kg orally) and/or TRH (50 mg/kg orally) in acute treatment. Control group (GI), TQ-treated group (GII), TRM-treated group (GIII), TRM+TQ-treated group (GIV). TQ, thymoquinone; TRM, tramadol.

Figure 2



Mean % changes in reaction time (s) in response to hotplate stimulus of TQ (20 mg/kg orally) and/or TRH (50 mg/kg orally) in chronic treatment. Control group (GI), TQ-treated group (GII), TRM-treated group (GIII), TRM+TQ-treated group (GIV). TQ, thymoquinone; TRM, tramadol.

Table 2 Mean % changes in the reaction time (s) to hotplate stimulus of thymoquinone (20 mg/kg) and/or tramadol (50 mg/kg orally) day 28 (chronic treatment)

Number (*N*=6)	Mean±SEM	*P* value
Control (GIa)	35±1.1	$P<0.001$
TQ treated (GIIa)	110±5.6	$P^{a}<0.001$
TRM treated (GIIIa)	181.33±6.3	$P^{a+b}<0.001$
TRM+TQ treated (GIVa)	310.2±9.1	$P^{c}<0.001$

TQ, thymoquinone; TRM, tramadol. P^{a}, significant difference between all-treated groups versus the control group, *P* value less than 0.001. P^{b}, significant difference between TRM-treated groups versus the TQ group, *P* value less than 0.001. P^{c}, significant difference between the combined TRM+TQ-treated groups versus either TRM-treated and/or the TQ-treated group.

(2) Chemical method.

An oral treatment of TQ (20 mg/kg orally) and/or TRM (50 mg/kg orally) suppressed the number of Writhing's (abdominal contractions) induced by an injection of acetic acid (10 mg/kg, intraperitoneally). However, a significant difference was observed on comparison between different groups versus the control group. Also, a comparison was performed between the different groups were found to be statistically significance (*P* less than 0.001) using ANOVA test (Table 3).

Antidepressant effect:

Effect of thymoquinone (20 mg/kg, orally) and/or tramadol (50 mg/kg orally) on the forced-swim test

An oral treatment of TQ (20 mg/kg orally) and/or TRM (50 mg/kg orally) or a combination of both induced a statistically significant increase in the swimming time and climbing time and a decrease in the immobility time compared with the control group ($P<0.001$). Also, comparative studies between TRM-treated and TQ-treated groups were found to be statistically significant ($P<0.001$).

However, of the combined TRM+TQ-treated group showed a significant increase in swimming and climbing, and decreased immobility time compared with either the TRM-treated or TQ-treated group (Tables 3 and 4).

Table 3 Effect of thymoquinone (20 mg/kg orally) and/or tramadol (50 mg/kg orally) on writhing in rats induced by an injection of acetic acid (10 mg/kg intraperitoneally)

Number (*N*=6)	Mean±SEM	Inhibition %	*P* value
Control (GIb)	20.33±2.10	0	$P<0.001$
TQ treated (GIIb)	16.12±0.91	19.67	$P<0.001$
TRM treated (GIIIb)	9.73±1.10	52.13	$P<0.001$
TRM+TQ treated (GIVb)	2.31±0.80	88.63	$P<0.001$

TQ, thymoquinone; TRM, tramadol.

Table 4 Effect of thymoquinone (20 mg/kg orally) and/or tramadol (50 mg/kg orally) on the forced-swim test: values are shown as mean±SEM

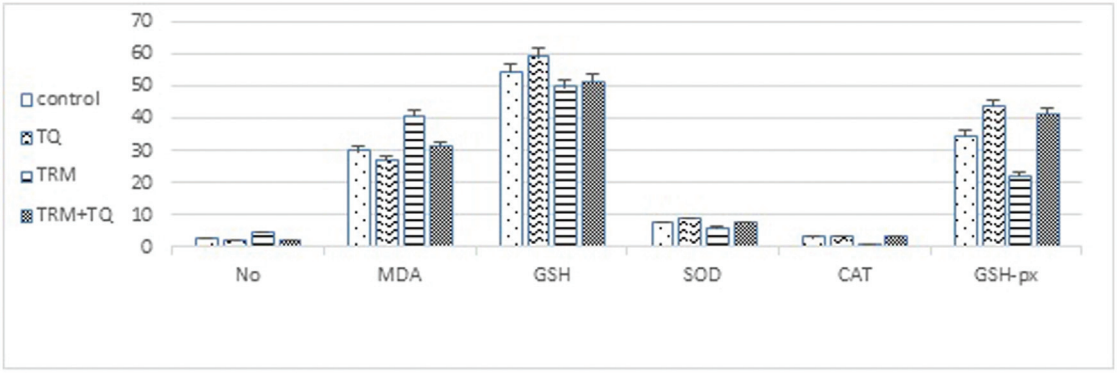
Number (*N*=6)	Swimming time (s) (mean ±SEM)	Climbing time (s) (mean ±SEM)	Immobility time (s) (mean ±SEM)	*P* value
Control (GIa)	110.51±5.3	10.21±0.9	78.50±3.11	$P<0.001$
TQ treated (GIIa)	142.4 ± 6.1^{a}	26.11 ± 1.9^{a}	41.15 ± 1.33^{a}	$P^{a}<0.001$
TRM treated (GIIIa)	168.3 ± 4.5^{ab}	48.12 ± 4.1^{ab}	28.16 ± 1.90^{ab}	$P^{a+b}<0.001$
TRM+TQ treated (GIVa)	$188.5\pm6.1^{a,c}$	56.51 ± 4.8^{ac}	18.8 ± 1.30^{ac}	$P^{a+c}<0.001$

Statistical analysis was carried out using analysis of variance. Treatment was continued for 27 successive days. TQ, thymoquinone; TRM, tramadol. P^{a}, significant difference between all groups versus the control group. P^{b}, significant difference between the TRM-treated groups versus the TQ group. P^{c}, significant difference between the combined TQ+TRM-treated groups versus either the TRM-treated or the TQ-treated group.

Biochemical results

Administration of TRM resulted in a significant decrease in whole-blood GSH content, activities of GSH-px, SOD, and CAT, and a significant increase in serum levels of MDA and NO compared with the

Figure 3



Effect of TRM administration and TQ supplementation on the content of GSH, the activities of GSH-px, SOD, and CAT and the levels of MDA and NO in different groups. CAT, catalase; GSH, glutathione; GSH-px, glutathione peroxidase; MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase; TQ, thymoquinone; TRM, tramadol.

normal control group. Supplementation of TQ with TRM induced a significant increase in the GSH content, activities of GSH-px, SOD, and CAT, and a significant reduction in the serum levels of MDA and NO compared with the group treated only with TRM Table 5.

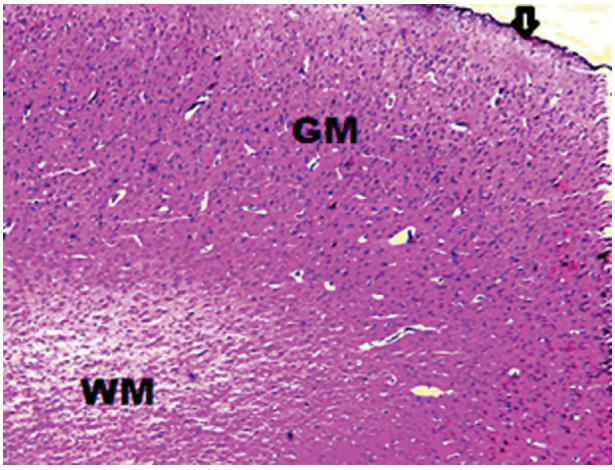
Histological results

Histological examination of H&E-stained sections of the cerebral cortex of GI rats showed a normal cerebral cortex structure, which was composed of outer gray and inner white matter; the latter only contained axons of nerve cells that were often myelinated (Figs 3 and 4).

The gray matter of the cerebral cortex is composed of nerve cells arranged in six layers lying just deep to the pia mater; these layers are as follows: (a) the outer molecular layer, which consists largely of fibers, and relatively few neuroglial cells. (b) The outer granular layer, which consists mainly of small pyramidal cells, and granular cells, also called stellate cells. (c) The outer pyramidal layer with pyramidal cells with open face nuclei, a basophilic cytoplasm, and long apical dendrites. (d) The inner granular layer, which has many small granular cells (stellate cells), but neuroglial cells were also prominent. (e) The inner pyramidal layer contains more pyramidal cells than layers II. (f) The layer of polymorphic cells contains cells with diverse shapes, many of which have a spindle or a fusiform shape. The ground substance between these nerve cells had a homogenous eosinophilic background called neuropil, which is composed of a network of branching cytoplasmic cell processes, axons, and dendrites (Figs 5 and 6).

Microscopic examination of sections of GII (TQ treated) cerebral cortex showed normal covering of

Figure 4

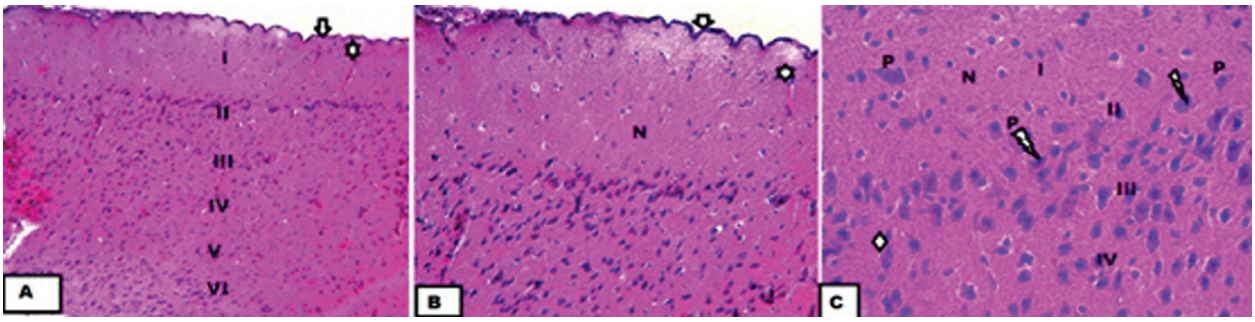


A photomicrograph of a section of the cerebral cortex (GI) showing a delicate layer of pia matter (arrow), outer gray matter (GM), and inner white matter (WM) (hematoxylin and eosin, ×40).

the pia matter. Its structure was more or less similar to that of the control; the pyramidal cell was observed to have a large rounded, basophilic cytoplasm, and processes; however, a few degenerated pyramidal cells were observed. Also, there were neuroglia cells with small dense nuclei and granular cells with open face vesicular nuclei and prominent nucleoli in the background of eosinophilic neuropil (Fig. 7).

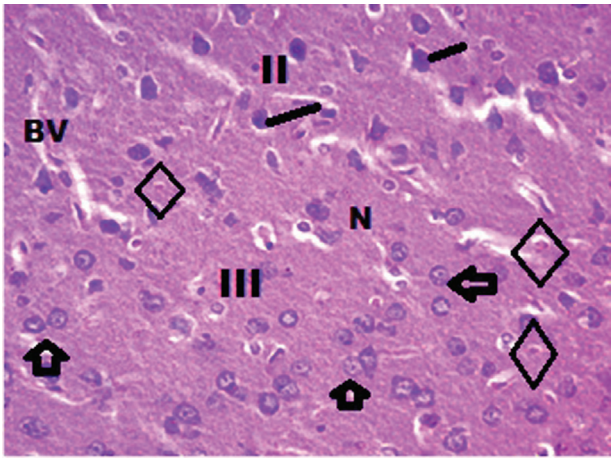
Histological examination of H&E-stained sections of the cerebral cortex from TRM –treated group GIII showed apparent thickening and cellular infiltration of the pia matter, marked disorganization, hypercellularity by mononuclear cell infiltration, and dilated blood capillaries of the cortical layers with the disorientation of the cortical layers compared with the control group (Fig. 8).

Figure 5



A photomicrograph of a section of the cerebral cortex from rats of GI showing: (a) a delicate layer of the pia matter (arrow) and six layers of the cortex: (I) outer molecular, (II) outer granular, (III)outer pyramidal, (IV) inner granular, (V) inner pyramidal, and (VI) polymorphic layer. Blood capillaries (star). (b, c) Higher magnifications of (a) showing pyramidal cells (P) with basophilic cytoplasm and vesicular nucleus (arrow heads), spindle-shaped cells (rectangles), and neuropil (N) (hematoxylin and eosin ×100, 200, and 400).

Figure 6



A photomicrograph of cerebral cortex section from rats of GI showing: layers II and III with pyramidal cells (/), granular cell with its vesicular nucleus (←) neuroglia cells inside the shape. Neuropil (N), blood vessels (BV) (hematoxylin and eosin ×400).

Examination of TRM –treated sections of the cerebral cortex showed increased vascularity bleeding, especially under the dura (subdural hemorrhage), and vacuolization of neuropil; most of the pyramidal cells appeared shrunken, irregular in shape, and darkly stained with pyknotic nuclei and many cells were surrounded by haloes (perineural space). Apoptotic cells increased, characterized by small hyperchromatic and pyknotic nuclei and chromatin condensation, which were detected in layers I, II, III, IV, V, and VI (Fig. 9).

Examination of the cerebral cortex of GIV TRM –treated and TQ-treated rats showed that the cerebral cortex layers regained their original arrangement, showed decreased vascularity, and a decrease in the number of apoptotic cells (Fig. 10).

Histological examination of the cerebral cortex of the cerebral cortex of rats stained with cresyl violet showed that the number of viable cells and the numbers of degenerated neurons had lightly stained nuclei with shrunken cell bodies (Fig. 11).

Immunohistochemical examination of cerebral cortex sections of the control and TQ-treated groups showed weak positive immune-expression for BAX compared with the TRM-treated group, and this reaction became weaker in the TRM+TQ-treated group (Fig. 12).

Histomorphometrical results

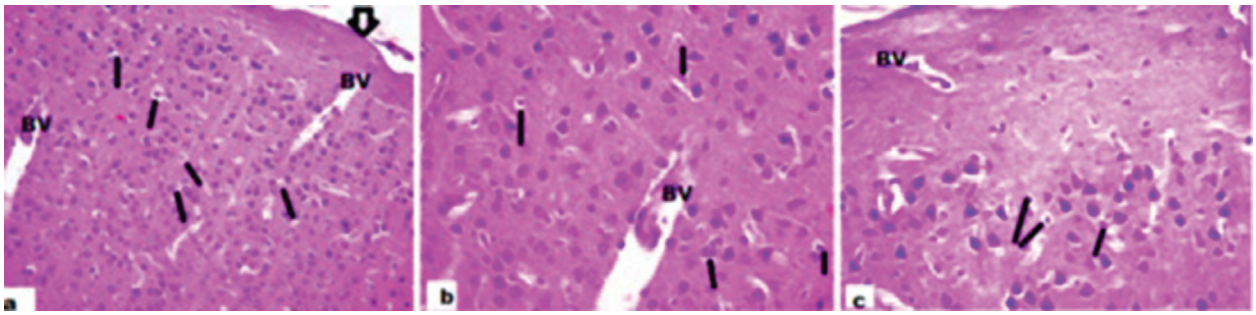
Administration of TRM resulted in a significant increase in the AI and the mean optical density of BAX immune-stained cerebral cortex compared with the control (GI) and (GII) TQ groups. Supplementation of TQ with TRM resulted in a significant decrease in the AI and optical density of BAX immuno-stain compared with the TRM –treated group (Table 6, Fig. 13).

Discussion

One of the most common prescription pain relievers is TRM, which is structurally similar to morphine and codeine, with its analgesic effects identified as a μ-receptor agonist. TRM was originally considered to have a much better safety profile than other opioid analgesics [1]. In the present study TQ alone exerted significant analgesic effects and improved the analgesic effect of TRM. Several mechanisms have been proposed to explain the analgesic effects of TQ. TQ exerts an anti-nociceptive effect through indirect activation of supraspinal and kappa-opioid receptor subtypes [45].

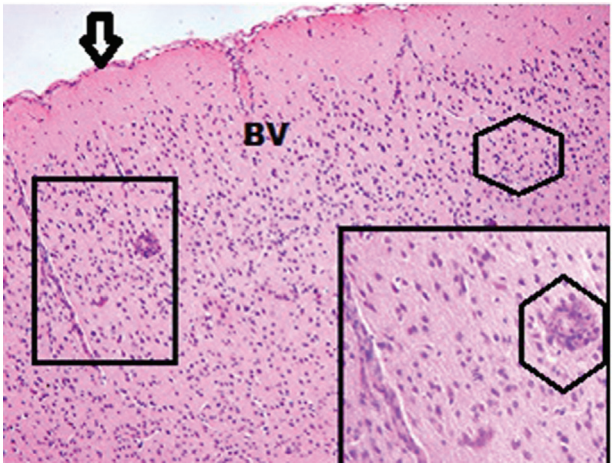
Beheshti *et al*. [46] reported that the analgesic effect of *N. sativa* could be because of its antioxidant, anti-inflammatory properties and microglia inhibitory

Figure 7



Photomicrographs of cerebral cortex sections of thymoquinone-treated rats of GII showing :(a) pia (↓), blood vessels (BV), normal pyramidal cells. Some pyramidal cells appear degenerated and surrounded by holes (*l*) (hematoxylin and eosin ×100, 200, and 400).

Figure 8



A photomicrograph of a section of the cerebral cortex from tramadol-treated rats of GIII showing: thickened pia (↓) with congested, dilated blood vessels (BV), disorientation of the layers with cellular infiltration inside shapes. The inset shows mononuclear cell infiltration (hematoxylin and eosin ×100).

activity of this plant. Another study by El-Dakhakhny *et al*. [47] showed that TQ can inhibit the generation of thromboxane A2 and leukotriene B4, suggesting an inhibitory effect on both cyclooxygenase and lipoxygenase pathways.

The finding of the present study showed that TRM alone caused anti-depressant-like behavior, which is probably because of increasing brain serotonin levels as TRM acts as a serotonin releaser and as a serotonin uptake inhibitor [48].

In the present study, TQ also exerted an anti-depressant effect and enhanced the anti-depressant effect of TRM. Perveen *et al*. [49] reported that the anti-depressant effect of TQ is achieved by increasing the 5 hydroxytryptamine (5-HT) concentration, which in turn modulates/downregulates the swim or hanging stress-induced serotonergic dysfunction. The synaptic enhancement of monoamine levels, predominantly 5-HT, is the key to anti-depressant effects.

It was reported that the administration of TQ led to an increase in the concentrations of 5-HT and 5-hydroxytryptophan (5-HTP) in the brain. In this respect, the anti-depressant-like effect of TQ appears to be modulated by an increase in the 5-HT concentration in the brain. In addition, *N. sativa*, through inhibition of acetylcholinesterase enzyme and particularly because of its antioxidative effects, improves diseases of the nervous system such as anxiety, depression, neurotoxicity, neurodegeneration, and pain [50].

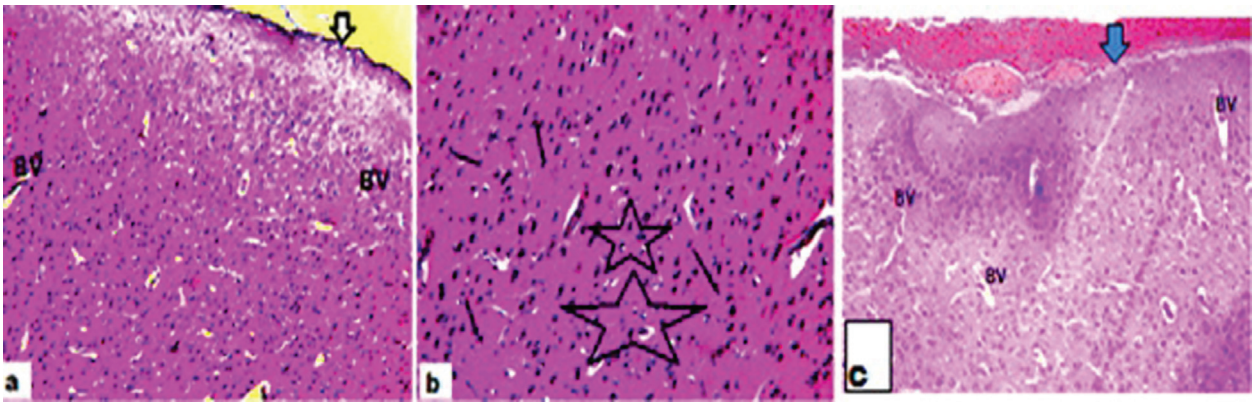
Gilhotra and Dhingra [51] also reported that TQ exerted its anti-depressant effect by producing a thiobarbituric acid-reactive substance and increasing reduced GSH levels. They suggested that TQ could be a potential candidate for the management of depression.

The present findings revealed a significant decrease in the GSH content and in the activities of SOD, CAT, and GSH-px in whole blood and a significant increase in the plasma level of MDA and NO of the TRM-treated group compared with the normal control and TQ only-treated groups. These findings are supported by previous researches performed on either *N. sativa* or its derivative TQ.

Oxidative stress occurs by an increase in lipid peroxidation and inhibition of the antioxidant enzyme activity [52]. MDA is widely accepted as a sensitive biomarker of lipid peroxidation [53] and a useful measure of oxidative stress status [54].

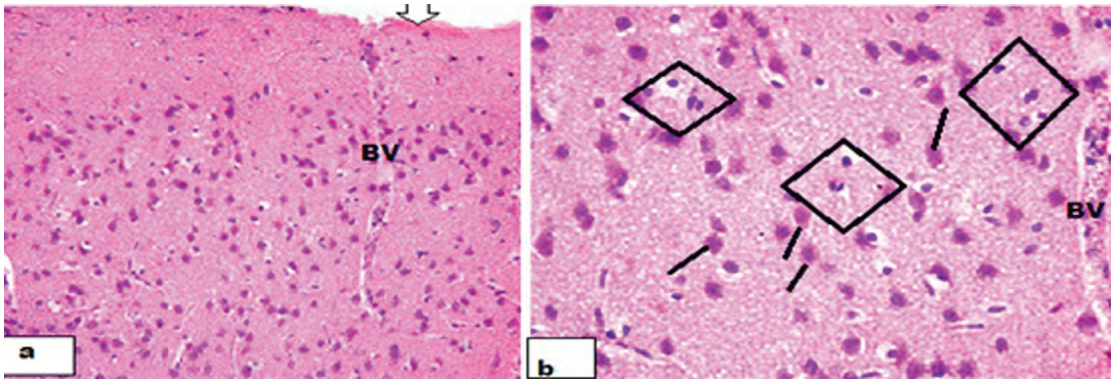
The previous study of El-Baky and Hafez [55] supported the hypothesis of decreased levels of SOD and reduced GSH in TRM-treated rats,

Figure 9



Photomicrograph of cerebral cortex sections from tramadol treated rats of GIII showing (a) thick pia (↓) with bleeding blood vessels (BV) hypercellularity with disorientation of the layers (b) showing many apoptotic cells (/) and cells degenerated surrounded by space inside shape (. . .), and (c) showed thick pia () and dilated blood vessels.

Figure 10



Photomicrographs of cerebral cortex sections of thymoquinone+tramadol-treated rats of GIV showing: (a) pia (↓), blood vessels (BV). (b) Normal pyramidal cells with its vesicular nucleus (/) and some degenerated by holes (◇). (hematoxylin and eosin ×100, 200).

which lead to enhanced H_2O_2 production, and stimulation of lipid peroxidation, NO, and protein oxidation. Ahmed and Kurkar [56] also reported that the administration of TRM increased the NO level and lipid peroxidation and decreased the antioxidant enzyme activity.

In addition, the present findings are supported by the previous study of Nna and Osim [57], who reported that the administration of TRM increased the level of MDA and decreased the levels of CAT, SOD and GSH-px in brain tissues. Reactive oxygen species (ROS) results in the formation of MDA (lipid peroxidation product). SOD, CAT, and GSH-px are known endogenous antioxidants quenching these ROS. Therefore, their decline after TRM use is an indicator of their consumption as a trial to compensate for oxidative stress [58].

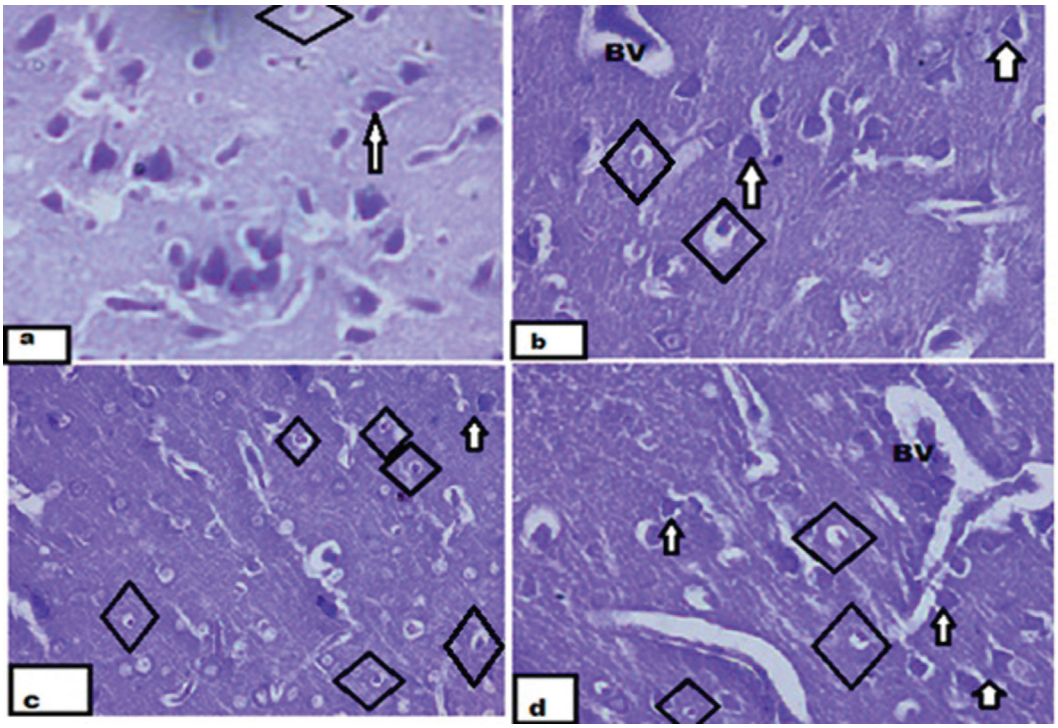
Decreased activity of the antioxidant enzymes by TRM could be attributed to the presence of a transition metal, as a cofactor, in the antioxidant enzymes. TRM interacted with a transition metal of these enzymes, resulting in inhibition of their activities [59].

The increased NO production may be because of the activation of the NO synthase-guanylate cyclase pathway by TRM, which was confirmed by a previous study by Song *et al.* [60].

The present results are also in parallel with those of Nehru and Anand [61], Abdel-Zaher *et al.* [62], and Elkhateeb *et al.* [63], who reported that a significant increase in the MDA level with a significant decrease or depletion in the GSH-px level in TRM –treated animals with subsequent potentiation of lipid peroxidation resulted in inhibition of protein synthesis and mitochondrial damage.

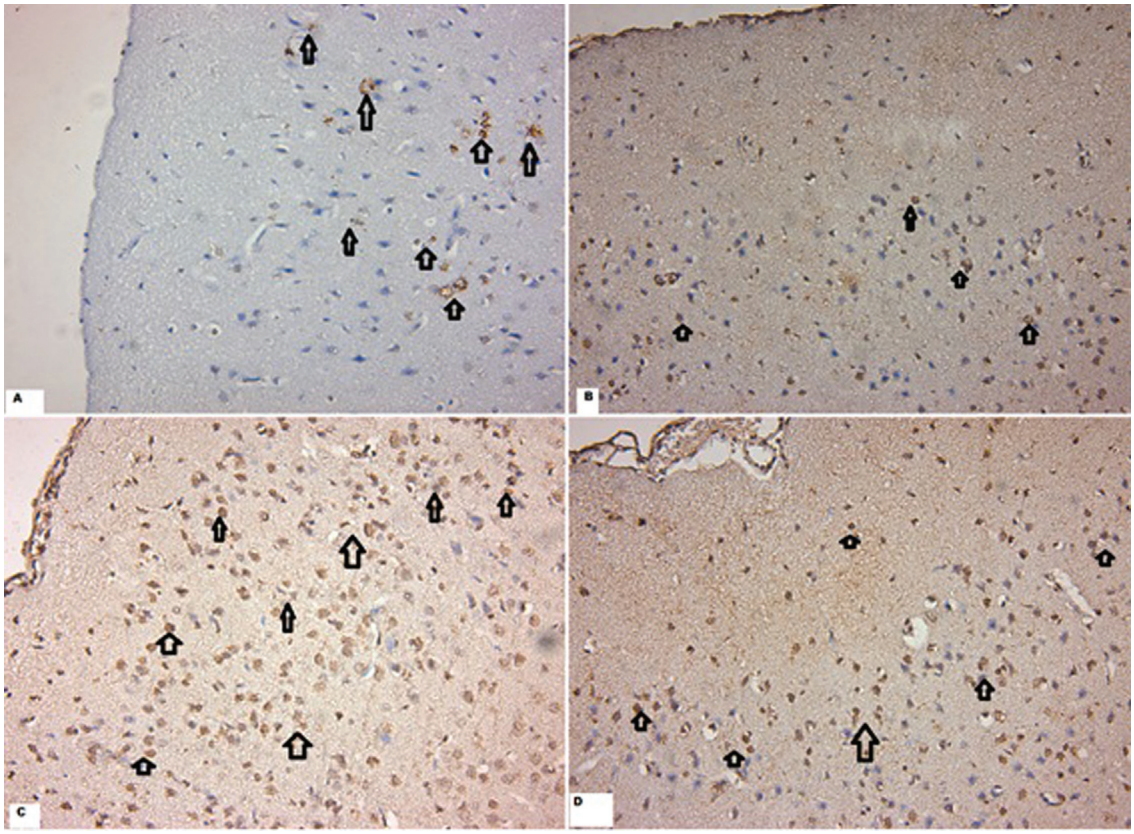
However, administration of TQ either alone or in combination with TRM induced a significant

Figure 11



Photomicrographs of cerebral cortex sections showing normal neurons (↑) and degenerated neurons (◊): (a) GI control, (b) GII thymoquinone (TQ), (c) GIII tramadol-treated, and (d) GIV tramadol+thymoquinone (cresyl violet ×400).

Figure 12



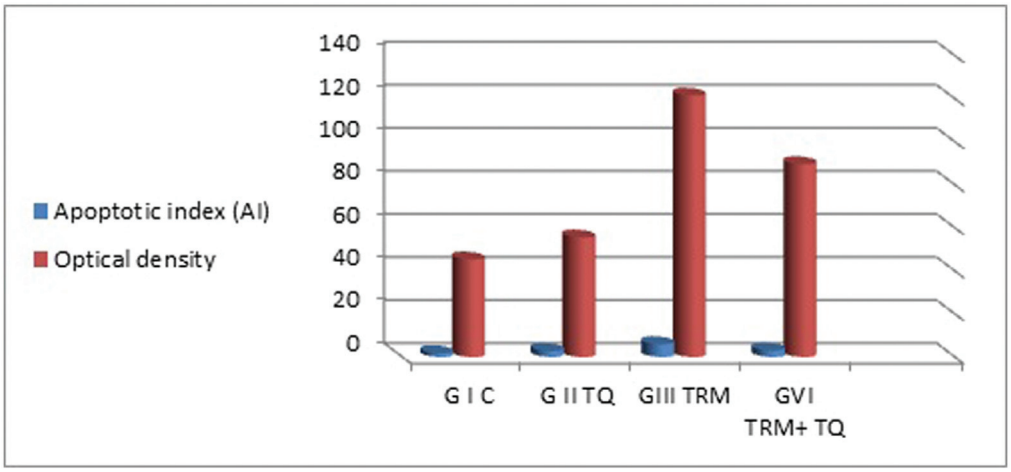
Photomicrographs of cerebral cortex sections showing immunoreaction for BAX (↑) in GI (a), moderate reaction in GII (b), strong reaction in GIII (c), and moderate reaction in GIV (d) (avidin–biotin peroxidase+H ×200).

Table 5 Effect of tramadol (50 mg/kg orally) and/or thymoquinone (10 mg/kg orally) on the changes in whole-blood glutathione content, superoxide dismutase activity, catalase activity, and glutathione peroxidase activity, and plasma malondialdehyde and nitric oxide

Number (*N*=6)	NO (μmol/l) (mean±SEM)	MDA (μmol/l) (mean±SEM)	GSH (mg%) (mean±SEM)	SOD (mg/ml of reaction mixture)	CAT (mg/dl)	GSH-px (mg GSH consumed/min/ml)	*P* value
Control (GI)	2.63±0.14	29.66±0.73	54.33±0.62	7.42±0.22	3.03 ±0.05	34.53±1.94	$P<0.001$
TQ treated (GII)	2.04±0.05[a]	27.02±0.78[a]	58.88±0.21[a]	8.70±0.25[a]	3.41 ±0.02[a]	43.73±1.59[a]	$P^a<0.001$
TRM treated (GIII)	4.63±0.21[b]	40.62±1.16[b]	49.60±0.83[b]	5.93±0.32[b]	0.93 ±0.03[b]	21.90±1.55[b]	$P^b<0.001$
TRM+TQ treated (GIV)	2.02±0.11[c]	30.93±0.42[c]	51.20±0.92[c]	7.29±0.25[c]	2.94 ±0.02[c]	41.35±2.12[c]	$P^c<0.001$

CAT, catalase; GSH, glutathione; GSH-px, glutathione peroxidase; MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase; TQ, thymoquinone; TRM, tramadol. P^a, significant difference between all treated groups versus the control group, *P* value less than 0.001. P^b, significant difference between TRM-treated groups versus the TQ group, *P* value less than 0.001. P^c, significant difference between combined TRM+ TQ-treated groups versus either the TRM-treated or the TQ-treated group.

Figure 1 3



Effect of TRM administration and TQ supplementation on apoptotic index and optical density of BAX immune-stain cerebral cortex cells' histochemical reaction in different groups. TQ, thymoquinone; TRM, tramadol.

increase in SOD, CAT, GSH, and GSH-px activities in whole blood and a significant decrease in the plasma levels of MDA and NO. These findings are in agreement with other studies that reported that TQ augments the antioxidant defense by preserving the activity of various antioxidant enzymes, such as CAT, GSH-px [64], and SOD [65]. TQ acts as a potent free radical and superoxide radical scavenger and it has anti-anxiety properties. It also inhibits the nonenzymatic lipid peroxidation in liposomes [66]. In addition, *N. sativa* could inhibit NO production and expression of inducible NO synthase as reported by Mansour *et al.* [67].

It was reported that the administration of TQ induced an increase in the concentration of 5-HT and 5-HTP in the brain. In this respect, the antidepressant-like effect of TQ appears to be modulated by increased 5-HT concentration in the brain. In addition, *N. sativa*, through inhibition of acetylcholinesterase enzyme and particularly because of its antioxidative effects, improves diseases of the nervous system such as anxiety, depression, neurotoxicity, neurodegeneration, and pain [50].

In the present study, supplementation with TQ attenuated the development of tolerance to the analgesic activity of TRM. This result is in agreement with Abdel-Zahar *et al.* [68], who reported that *N. sativa* oil appears to have therapeutic potential in TRM tolerance and dependence through the blockage of NO overproduction and oxidative stress induced by the drug.

The current study showed that the histological alteration of TRM-treated cortex includes cortical layers' disorganization, hypercellularity, dilated blood vessels, increased apoptotic cells as well as extensive neuropil vacuolization, which was similar to the results of a study carried out by Ghoneim *et al.* [26].

Table 6 Apoptotic index and the mean optical density of the BAX immunohistochemical reaction in the cerebral cortex of all studied groups

Number (*N*=6)	Apoptotic index (mean±SEM)	Optical density (mean±SEM)	*P* value
Control (GI)	1.84±0.47	45.4±5.442	$P<0.001$
TQ treated (GII)	2.832±7.05[a]	55.6±5.973[a]	$P^a<0.001$
TRM treated (GIII)	6.05±2.05[b]	121.5±13.72[b]	$P^b<0.001$
TRM+TQ treated (GIV)	3.072±0.653[c]	89.654±7.94[c]	$P^c<0.001$

TQ, thymoquinone; TRM, tramadol. P^a, significant difference between all treated groups versus the control group, *P* value less than 0.001. P^b, significant difference between TRM-treated groups versus the TQ group, *P* value less than 0.001. P^c, insignificant difference between the combined TRM+TQ-treated groups versus either the TRM-treated or the TQ-treated group, *P* value less than 0.05.

These changes could be attributed to induction of oxidative stress by TRM as supported by the findings of Omar [69], who reported that the administration of TRM induces oxidative stress through its inflammatory reaction and a significant decrease in the antioxidants in brain tissue. The oxidative stress induced by TRM in the brain was also reported by Mohamed *et al*. [70] and Nafea *et al*. [71].

TRM induces inflammatory reactions that may be the cause of oxidative stress by altering cell membrane fatty acid composition, leading to a decrease in its fluidity, which in turn will impair the formation of pseudopodia and internalization of pathogens and foreign particles [72]. The brain is highly susceptible to oxidative damage because of its high oxygen consumption, high polyunsaturated fatty acid concentration, and low levels of antioxidants. Oxidative stress causes modifications of the brain protein that result in loss of function and decrease in enzyme activity [73].

Dal *et al*. [74] reported that NO is involved in the analgesic effect of TRM; the reaction between NO and superoxide yields peroxynitrite, which exerts a toxic effect through its direct oxidative mechanisms. Nitration of structural proteins such as neurofilaments and actin can disrupt filament assembly and induce pathological consequences, especially with long-term use of opioids, which can induce neuronal damage and affect the neuronal cytoskeleton.

Ahmed and Kurkar [56] attributed the histological changes induced by the use of TRM to its ability to generate oxygen free radicals, which can attack the cell membrane as a result of lipid peroxidation and lead to destabilization and disintegration of the cell membrane.

In the current study, all TRM-treated animals showed congestion of the brain and submeningeal congestion of blood vessels and neural degeneration. These results are similar to those of Jana *et al*. [75], who reported that TRM may affect the endothelial cells of brain vessels, resulting in the release of NO, which has an endothelium-relaxing effect, leading to vascular congestion.

In this study, apoptosis was observed in the cerebral cortex of TRM-treated rats by light microscopic examination; degenerated pyramidal cells appeared either darkly stained with pyknotic nuclei or with faintly stained cytoplasm. Some pyramidal cells were shrunken and showed marked cytoplasmic vacuolization. This was in agreement with the result reported by Ghoneim *et al*. [26], who concluded that chronic use of TRM is found to cause red neuron degeneration and apoptosis in the rat brain, which probably contributes toward the cerebral dysfunction because of oxidative stress in this organ. The vacuolation could be attributed to the damaged cell organoid from exposure to free radicals as reported by Liu *et al*. [76].

That was in agreement with Lei *et al*. [77] and Wajid *et al*. [78], who concluded that TQ acts by generating ROS, which induces low expression of prosurvival genes and conformational changes in pro-apoptotic proteins, resulting in loss of mitochondrial membrane potential. This leads to the activation of caspase-9, caspase-3, and polyadenosine 5'-diphosphate ribose polymerase cleavage and caspase-dependent apoptosis.

Liu *et al*. [76] also reported that the multiple effects of opioids on neuronal structure (cytoskeleton) and neuronal damage such as TRM induce mRNA expression of pro-apoptotic receptors in the lymphocytes, spleen, lung, and heart by activating an opioid receptor. Also NO, decreased myelin-associated glycoprotein, or autoantibodies to myelin basic protein that cause the neuronal damage.

In this study, a marked reduction in cellular and apoptotic changes was observed in the cerebral cortex of TRM+TQ-treated rats. These results were in agreement with those of Mousa *et al*. [79].

The neuroprotective role of TQ may be because of its action against oxidative damage induced by free

radicals of TRM that generated the histological changes. The antioxidant activity of TQ is because of inhibition of thromboxane B2 and leukotriene B4 and thus prevents membrane lipid peroxidation in the tissues [80]. Furthermore, Radad *et al*. [81] attributed the protective effect of TQ to its anti-inflammatory and immunomodulatory protective effects against TRM-induced cellular damage.

Conclusion

In conclusion, TQ potentiates the analgesic and anti-depressant effects of TRM. Moreover, TQ protects against the development of tolerance, oxidative stress, and neurotoxicity, and changes in the cerebral cortex induced by TRM. Therefore, the combination of both TRM and TQ decreases the dose of TRM and avoids undesirable side effects. Further studies are needed to evaluate the effect of clinical uses of TQ on the nervous system.

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Nil.

Conflicts of interest

None declared.

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