Citicoline Protects against Tramadol-Induced Oxidative Stress and Organ Damage

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ABSTRACT | Tramadol is a common drug of abuse which has been shown to cause neurodegeneration in the rat brain. Cytidine-5'-diphosphocholine or citicoline is an intermediate in the synthesis of phosphatidylcholine and is in use in humans for the treatment of several brain pathologies. In this study, we aimed to investigate the effect of citicoline on oxidative stress and tissue injury caused by tramadol, an opioid drug. Rats were treated with tramadol at 30 mg/kg alone or in combination with citicoline at 50, 100, or 200 mg/kg orally, once a day, for 10 days. Other groups were treated with only 0.9% saline or only citicoline at 200 mg/kg. Lipid peroxidation (malondialdehyde), nitric oxide, reduced glutathione (GSH), and paraoxonase-1 (PON-1) activity were measured in the serum. Bone marrow DNA fragmentation assay and micronucleus test were also done. In addition, histopathological examination of the brain, liver, and kidney, and immunohistochemical staining for glial cell acidic fibrillary protein (GFAP) in the cerebral cortex were performed. Results indicated that compared to the saline-treated group, repeated tramadol administration led to significant increases in serum malondialdehyde and nitric oxide concentrations by 50.0% and 70.0%, respectively. There was also a decline in GSH content and PON-1 activity in the serum by 26.3% and 51.4%, respectively. Tramadol caused marked DNA fragmentation and increased the number of micronucleated polychromatic erythrocytes (MnPCE) in bone marrow by 192.8% and 876.3%, respectively, compared with the corresponding saline control values. Histopathological studies revealed neuronal degeneration (acidophilic cytoplasm and dark nuclei) and decreased GFAP immunostaining in the cerebral cortex of tramadol-treated rats. The liver exhibited fibrosis, apoptotic hepatocytes, and inflammatory cell infiltration. Vacuolar degeneration of the tubular lining epithelium and edema of glomeruli were observed in the kidney. Citicoline administered to saline-treated rats at a dose of 200 mg/kg showed no significant effect on serum malondialdehyde, nitric oxide, GSH concentrations, or PON-1 activity compared with the saline control group. Citicoline by itself had no effect on DNA fragmentation or the number of MnPCE in the bone marrow. In tramadol-treated rats, however, citicoline (50–200 mg/kg) resulted in a significantly decreased malondialdehyde by 23.8%–31.6%. Nitric oxide decreased by 29.2%–36.2% after citicoline at 50–200 mg/kg. There was also a significant increase in both GSH by 19.6%–33.6% and in PON-1 activity by 54.8%–125.7%. In addition, citicoline caused a significant decrease in DNA fragmentation (by 29.2%–52.4%) and the number of MnPCE in the bone marrow (by 20.5%–59.5%) in tramadol-treated rats. Histopathological changes caused by tramadol in the brain, liver, and

kidney were ameliorated by treatment with citicoline. We conclude that citicoline treatment results in decreased lipid peroxidation and nitric oxide along with increased GSH content and PON-1 activity in the serum of rats treated with tramadol. These changes might account for the beneficial effect of citicoline in decreasing DNA fragmentation and tissue injury caused by tramadol.

KEYWORDS | Citicoline; DNA fragmentation; Lipid peroxidation; Micronucleus test; Oxidative stress; Paraoxonase-1; Tramadol

ABBREVIATIONS | GFAP, glial cell acidic fibrillary protein; GSH, reduced glutathione; MDA, malondialdehyde; MnPCE, micronucleated polychromatic erythrocyte; NOS, nitric oxide synthase; PON-1, paraoxonase-1

CONTENTS

1. INTRODUCTION

Tramadol is a synthetic 4-phenylpiperidine analog of codeine. Following oral administration, the drug is readily absorbed and metabolized in the liver. The active metabolite is desmethyltramadol [1, 2]. The agent is a potent centrally acting analgesic that is being widely used to alleviate moderate to severe pain caused by musculoskeletal disorders or cancer [3, 4]. It is a weak mu-receptor opioid agonist and, in addi-

tion, inhibits the neuronal re-uptake of serotonin, noradrenaline, and dopamine [1, 2, 5] and appears to have antidepressant properties [6]. The latter effect besides its potent analgesic properties might also contribute to its continued use by patients after improvement of their musculoskeletal problems [7, 8]. Reports of tramadol dependence and/or abuse represent a growing health concern worldwide [9–13]. In Egypt, the drug is included in the narcotic analgesic schedule III [13]. Very few studies have examined the neurotoxic potential of tramadol, but the drug has been found to cause comorbid anxiety, and depressive as well as obsessive-compulsive symptoms in humans when taken at high doses (~675 mg or more) for several years [14]. Serotonin syndrome [15], generalized tonic clonic seizures [16] and cognitive impairment [17] have also been reported. Studies in rats showed that the repeated administration of tramadol caused seizures and the appearance of red neurons in the brain, indicating the development of neurodegeneration [18]. Oxidative stress has been implicated in the development of neurodegenerative disorders like Parkinson's disease, Alzheimer's disease, and in aging [19, 20]. Oxidative stress ensues when the increase in the generation of reactive oxygen and other free radical species overcomes the capacity of the cell's antioxidants with resultant damage to the cell membrane lipids, proteins, and DNA [21]. Oxidative stress occurs in the rat brain after tramadol where increased lipid peroxidation (malondialdehyde) and decreased activities of the antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase as well as decreased levels of reduced glutathione (GSH) have been detected [22, 23]. Oxidative stress might thus contribute to the tramadol neurotoxicity.

Cytidine-5′-diphosphocholine or CDP-choline is an intermediate in the biosynthesis of the major brain phospholipid phosphatidylcholine and other phospholipids as well, including phosphatidylserine and phosphatidylethanolamine. It is composed of cytidine and choline which are linked by a diphosphate bridge. When taken orally, CDP-choline is hydrolyzed and absorbed as cytidine and choline, distributed across the body and readily crosses the blood-brain barrier [24, 25]. It is largely thought that inside brain cells, re-synthesis of CDP-choline occurs which results in increased phospholipid synthesis following intake of CDP-choline [26]. Citicoline, is an international non-proprietary name of cytidine-5'-

diphosphocholine and is in use in several countries for the management of various brain disorders such as Alzheimer's and Parkinson's diseases, traumatic brain injury, cerebrovascular disease, and cognitive decline in aging [24–26]. Citicoline has been shown to exert neuroprotective effects in experimental models of brain injury, reducing brain edema and infarct size, restoring the activity of membrane Na^+/K^+ ATPase [27] and mitochondrial ATPase [28] and inhibiting the activation of phospholipase A2 [29].

In this study, we aimed to investigate the ability of CDP-choline or citicoline to protect against oxidative stress caused by tramadol. Moreover, the effect of treatment with tramadol given alone or in conjunction with citicoline on the brain, liver and kidney was examined using histopathological and immunostaining for glial cell acidic fibrillary protein (GFAP) in the cerebral cortex. Bone marrow DNA fragmentation assay and micronucleus test were also performed.

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague-Dawley rats, weighing 180–200 g, were group-housed under temperature- and light-controlled conditions. Rats were allowed standard laboratory rodent chow and water ad libitum. The study followed the recommendations of the Institutional Animal Ethics Committee and conducted in accordance with the guidelines of the United States National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and Chemicals

Citicoline (cytidine 5′-diphosphocholine) available as Somazina® 100 mg/ml oral solution, was from Ferrer Internacional SA, Barcelona, Spain. Tramadol was a kind gift from the Ministry of Justice, Egypt. Drugs were dissolved in physiological saline to obtain the necessary doses.

2.3. Study Design

Rats were randomly divided into six groups with 6 animals in each group. The following groups were studied: Group 1, treated with 0.9% saline; Group 2,

treated with 200 mg/kg citicoline; Group 3, treated with 30 mg/kg tramadol; Group 4, treated with 30 mg/kg tramadol + 50 mg/kg citicoline; Group 5, treated with 30 mg/kg tramadol + 100 mg/kg citicoline; and Group 6, treated with 30 mg/kg tramadol + 200 mg/kg citicoline. Tramadol and citicoline were given orally at the same time. Treatments were given daily for 10 days. At the end of the experiments, blood samples were obtained from the retro-orbital venous plexuses under ether anesthesia. Rats were then euthanized under light ether anesthesia for tissue collection.

2.4. Biochemical Analyses

2.4.1. Lipid Peroxidation

Lipid peroxidation products in the serum were assayed by measuring the level of malondialdehyde (MDA) using the method of Ruiz-Larrea et al. [30] where the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having a peak absorbance at 532 nm.

2.4.2. GSH

GSH was determined using the method of Ellman et al. [31]. The procedure is based on the reduction of Ellman′s reagent by –SH groups of GSH to form 2-nitro-5-mercaptobenzoic acid, which is intense yellow in color and determined using a spectrophotometer at 412 nm.

2.4.3. Nitric Oxide

Nitric oxide was determined using the Griess reagent, according to the method of Moshage et al. [32]. Nitrite, a stable end product of nitric oxide radical, is mostly used as an indicator for the production of nitric oxide. Nitrate is converted to nitrite via nitrate reductase. The Griess reagent then acts to convert nitrite to a deep purple azo compound which can be determined using a spectrophotometer.

2.4.4. Paraoxonase-1 Activity

Arylesterase activity of paraoxonase-1 (PON-1) was measured using phenylacetate as a substrate. PON-1 catalyzes the cleavage of phenyl acetate resulting in phenol formation, the rate of which is measured by monitoring the increase in absorbance at 270 nm at 25°C using a spectrophotometer. One unit of arylesterase activity is defined as 1 mmol of phenol formed per min. Enzyme activity was calculated based on phenol's extinction coefficient of 1,310 M^{-1} cm^{-1} at 270 nm, pH 8.0, and 25°C, and expressed as kilo international units/liter (kU/L) [33].

2.5. DNA Fragmentation by Diphenylamine Assay

The bone marrow from the femurs of rats was flushed out with 1× Hank's balanced buffer solution (HBSS) into a centrifuge tube. The cells were collected by centrifugation at 500 g for 10 min. Cell pellets were re-suspended with 1× HBSS containing 2% fetal bovine serum (FBS). Then, the bone marrow cellularity was determined. DNA fragmentation in the bone marrow cells were carried out according to Zhivotovsky et al. [34]. Briefly, 2×10^6 cells were lysed in lysis buffer (5 mM Tris-HCl, pH 8.0, containing 20 mM EDTA and 0.5% Triton X-100) for 30 min at 4°C. The cell lysate was centrifuged at 15,000 g for 15 min at 4°C. Then, the supernatant containing small DNA fragments was separated from the pellet containing large pieces of DNA. The supernatant and pellet were re-suspended in 10 and 5% of trichloroacetic acid, respectively, and kept overnight. Then both samples were heated at 95°C for 15 min and centrifuged at 2,500 g for 5 min to remove proteins. Supernatant fractions were reacted with diphenylamine for 24 h at 37°C and the developing blue color was measured at 600 nm. DNA fragmentation in samples was expressed as percentage of total DNA appearing in the supernatant fraction using the formula: DNA fragmentation = [OD of fragmented DNA (S)] ÷ [OD of fragmented DNA (S) + OD of intact DNA (P)] ×100, where OD, S, and P denote optical density, supernatant, and pellet, respectively.

2.6. Micronucleus Test

Bone marrow slides were prepared according to the method described by Krishna and Hayashi [35]. The bone marrow was washed with 1 ml of FBS and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 min, followed by staining in May-Grunwald-Giemsa for 5 min then washed in distilled water and mounted. For each animal, 2,000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei.

2.7. Histopathological Studies

The brain, liver and kidney samples of all animals were dissected immediately after euthanasia. The specimens were then fixed in 10% neutral-buffered formalin saline for at least 72 h. All the specimens were washed in tap water for 30 min and then dehydrated in ascending grades of alcohol, cleared in xylene, and embedded in paraffin. Serial sections of 6 µm thickness were cut and stained with hematoxylin and eosin (H&E) for histopathological investigation. Images were examined and photographed under a digital camera (Microscope Digital Camera DP70, Tokyo, Japan), and processed using Adobe Photoshop version 8.0.

2.8. Immunohistochemistry for Glial Fibrillary Acidic Protein

Paraffin-embedded brain sections were deparaffinized and hydrated. Immunohistochemistry was performed with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) for detection of the GFAP activity. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with anti-GFAP antibodies (1:50 dilution) overnight at 4°C. After washing with phosphate-buffered saline (PBS), followed by incubation with biotinylated goat-anti-rabbit- immunoglobulin G secondary antibodies (1:200 dilution; Dako) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako/Agilent, Santa Clara, CA, USA) for 30 min at room temperature, and the binding sites of antibody were visualized with diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA). After washing with PBS, the samples were counterstained with H&E for 2–3 min and dehydrated by transferring them through increasing ethanol solutions (30, 50, 70, 80, 95, and 100% ethanol). Following dehydration, the slices were soaked twice in xylene at room temperature for 5 min, mounted, examined, and evaluated by a high-power light microscope.

2.9. Morphometric Studies

Detection of GFAP percentage area was carried out using the Leica Qwin 500 Image Analyzer (LEICA, Cambridge, UK), which consists of a Leica DM-LB microscope with a JVC color video camera attached to a computer system Leica Q 500IW at the Pathology Department, National Research Center (Cairo, Egypt). The GFAP area was determined as an area per field in micrometer square, area fraction, and area percentage by using the interactive software of the system. The area was measured in 10 fields in each slide.

2.10. Statistical Analysis

Statistical analysis was performed with SPSS software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's post hoc test for comparison between different treatments. Results were reported as mean ± SE, and differences were considered as significant when $p < 0.05$.

3. RESULTS

3.1. Biochemical Results of Oxidative Stress

3.1.1. Lipid Peroxidation

The serum MDA concentration in rats treated with citicoline at 200 mg/kg did not differ significantly from the saline control value. The repeated tramadol administration led to a significantly higher serum MDA level by 55.0% compared to the saline group (77.66 ± 3.36 vs. 50.1 ± 1.44 µmol/L). Significant decreases in the MDA concentration by 23.8% and 31.6% were observed in rats given tramadol and citicoline at 100 and 200 mg/kg, respectively, as compared to the tramadol only-treatment group (**Table 1**).

3.1.2. Nitric Oxide

Citicoline given at 200 mg/kg showed no significant effect on serum nitric oxide compared to the saline control group. Nitric oxide in the serum, however, showed a significant increase by 70% in tramadol-treated rats compared to the saline treated group. This increase in nitric oxide decreased by 29.2%, 30.0%, and 36.2% after treatment with citicoline at 50, 100, and 200 mg/kg, respectively (**Table 1**).

3.1.3. GSH

No significant difference in the concentration of GSH in the serum between rats given only citicoline

TABLE 1. MDA, nitric oxide, and GSH levels, and PON-1 activity in the serum of rats treated with citicoline, or tramadol, or both

Treatment	MDA (µmol/L)	Nitric oxide (µmol/L)	GSH (µmol/L)	PON-1 (kU/L)
Saline	50.10 ± 1.44	33.8 ± 1.4	22.34 ± 0.85	170.35 ± 3.70
Citicoline 200 mg/kg	52.30 ± 1.28	30.4 ± 0.76	20.82 ± 0.66	189.12 ± 7.29
Tramadol 30 mg/kg	$77.66 \pm 3.36^*$	$57.44 \pm 1.9^*$	$16.46 \pm 0.67^*$	$82.73 \pm 4.18^*$
Tramadol + citicoline 50 mg/kg	$68.15 \pm 1.15^*$ (−12.2%)	$40.68 \pm 1.70^{*+}$ (−29.2%)	$18.32 \pm 0.95^*$ (11.3%)	$128.1 \pm 9.64^*$ (54.8%)
Tramadol + citicoline 100 mg/kg	$59.15 \pm 2.52^+$ (−23.8%)	$40.20 \pm 2.21^{*+}$ (−30.0%)	$19.68 \pm 0.78^+$ (19.6%)	$169.31 \pm 7.79^+$ (104.6%)
Tramadol + citicoline 200 mg/kg	$53.10 \pm 0.94^+$ (−31.6%)	$36.64 \pm 1.74^+$ (−36.2%)	$22.00 \pm 1.14^+$ (33.6%)	$186.7 \pm 7.92^+$ (125.7%)

Note: *, $p < 0.05$ vs. corresponding saline-treated group; +, $p < 0.05$ vs. tramadol only group; #, $p < 0.05$ vs. tramadol + citicoline at 50 or 100 mg/kg treatment groups. The percent change from the tramadol group is shown in parentheses.

TABLE 2. Effect of tramadol and citicoline on DNA fragmentation in bone marrow cells of rats

Treatment	% DNA fragmentation
Saline	2.21 ± 0.16
Citicoline 200 mg/kg	2.11 ± 0.18
Tramadol 30 mg/kg	$6.47 \pm 0.29^*$
Tramadol + citicoline 50 mg/kg	$5.72 \pm 0.34^*$ (−11.6%)
Tramadol + citicoline 100 mg/kg	$4.58 \pm 0.23^{*+}$ (−29.2%)
Tramadol + citicoline 200 mg/kg	$3.08 \pm 0.16^{*+}$ (−52.4%)

Note: *, $p < 0.05$ vs. corresponding saline-treated group; +, $p < 0.05$ vs. tramadol only group. The percent change from the tramadol group is shown in parentheses.

at 200 mg/kg or saline. Serum GSH, however, fell by 26.3% in rats treated with tramadol compared with the saline group. Citicoline given at 100 and 200 mg/kg resulted in 19.6% and 33.7% increments in GSH compared with the tramadol only group, respectively (**Table 1**).

3.1.4. PON-1 Activity

Citicoline given at 200 mg/kg had no significant effect on serum PON-1 activity compared to the saline control group. On the other hand, PON-1 activity showed a significant decrease by 51.4% after tramadol administration. Citicoline given at 50, 100, and 200 mg/kg resulted in 54.8%, 104.6%, and 125.7% increments in PON-1 activity compared with the tramadol only group, respectively (**Table 1**).

3.2. DNA Fragmentation

Citicoline given alone had no significant effect on DNA fragmentation in cells of bone marrow. In contrast, tramadol induced a significant increase in DNA fragmentation by 192.8% compared with the saline control value. DNA fragmentation decreased significantly by 29.2% and 52.0% after treatment with citicoline at 100 and 200 mg/kg, respectively (**Table 2**).

3.3. Micronucleated Polychromatic Erythrocytes

There was no significant effect for citicoline alone at 200 mg/kg on the number of micronucleated polychromatic erythrocytes (MnPCE) in the bone marrow. A significant increase in MnPCE by 876.3% was observed in the rats given tramadol compared with the

TABLE 3. Effect of tramadol and citicoline on the frequencies of MnPCE (micronuclei per 2000 PCE) in the bone marrow cells of rats

Treatment	MnPCE
Saline	3.50 ± 0.12
Citicoline 200 mg/kg	2.67 ± 0.11
Tramadol 30 mg/kg	$34.17 \pm 0.21^*$
Tramadol + citicoline 50 mg/kg	$27.17 \pm 0.15^*$ (−20.5%)
Tramadol + citicoline 100 mg/kg	$21.33 \pm 0.12^{*+}$ (−37.6%)
Tramadol + citicoline 200 mg/kg	$13.83 \pm 0.07^{*+\#}$ (−59.5%)

Note: *, $p < 0.05$ vs. corresponding saline-treated group; +, $p < 0.05$ vs. tramadol only group; #, $p < 0.05$ vs. tramadol + citicoline at 50 or 100 mg/kg treatment groups. The percent change from the tramadol group is shown in parentheses.

saline control value. The increase in MnPCE by tramadol was significantly decreased by 37.6% and 59.5% after the administration of 100 and 200 mg/kg citicoline, respectively (**Table 3**).

3.4. Histopathological and Immunohistochemical Results

3.4.1. Brain Tissue

Sections from saline-treated rats showed the normal histological structure (**Figure 1A**). Repeated administration of tramadol resulted in signs of degeneration to appear in many neurons of the cerebral cortex tissue (**Figure 1B**). Citicoline had an ameliorating effect on tramadol- induced damage that was dose-dependent (**Figure 1C–1E**). Citicoline given alone at 200 mg/kg had no effect (**Figure 1F**).

Examination of brain tissue sections stained immunohistochemically with GFAP antibody further supports the results of histopathological examination. Tramadol decreased markedly the positive reaction of the stain due to a decrease in glial cell number and/or size. This effect was ameliorated by citicoline (**Figure 2**).

Further histopathological evaluation was done using quantitative morphometric analysis of GFAP which is detected in the cytoplasm of viable astrocytes. It was marked by the blue color in the image analyzer system to be measured as an area%. The maximum expression ($4.87 \pm 0.31\%$) was in the normal control group which received saline only. On the other hand, the lowest GFAP expression ($0.88 \pm 0.4\%$) was noted in the group which was treated with tramadol only, indicating its destructive effect on

neuronal tissue. The area% of GFAP increased gradually by treatment with citicoline. Significant increments in area% of GFAP were observed in groups which received tramadol combined with citicoline compared to the tramadol only group (**Table 4**).

3.4.2. Liver Tissue

Rats treated with saline showed normal liver histology (**Figure 3A**). Following tramadol administration, enhanced apoptosis was seen in many hepatocytes as well as fibrosis in between (**Figure 3B**). Citicoline reduced these effects in a dose-dependent manner (**Figure 3C–3E**). Citicoline alone had no effects (**Figure 3F**).

3.4.3. Kidney Tissue

Rats treated with saline showed normal structure of glomeruli and tubules (**Figure 4A**). Tramadol caused marked vacuolar degeneration of the tubular lining epithelium and glomerular edema (**Figure 4B**). These changes were ameliorated by treatment with citicoline (**Figure 4C–4E**). Citicoline given alone had no effect on kidney tissue (**Figure 4F**).

4. DISCUSSION

In this study, we investigated the effect of repeated doses of tramadol, a known drug of abuse, on systemic oxidative stress and DNA fragmentation and on the brain, liver, and kidney histology. We, in addition, examined the potential protective effect of CDP-choline or citicoline. Our results provide evi-
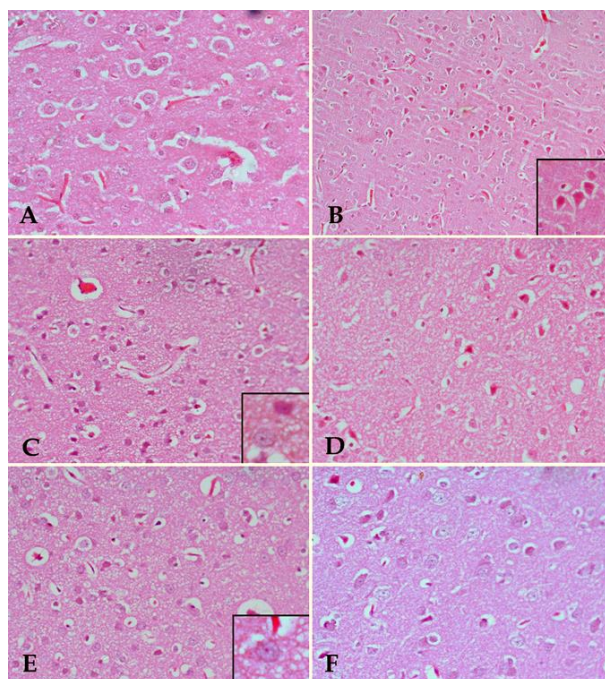
FIGURE 1. Representative photomicrographs of cerebral cortex sections from various groups. (A) Saline: normal structure of the tissue with normal shaped neurons. (B) Only tramadol: many neurons with signs of degeneration (acidophilic cytoplasm and dark nuclei) among normal neurons. (C) Tramadol + citicoline 50 mg/kg: no remarkable reduction of damaged cells was observed. (D) Tramadol + citicoline 100 mg/kg: noticeable reduction of neurons with signs of degeneration. (E) Tramadol + citicoline 200 mg/kg: only a few cells with signs of degeneration. (F) Only citicoline 200 mg/kg: more or less normal cerebral cortex structure. H&E staining.

FIGURE 2. Representative photomicrographs of cerebral cortex tissue sections stained immuno-histochemically with GFAP antibody after various treatments. (A) Saline: normal reaction of the stain; the upper right part of figure shows the normal shape of glial cells (body and processes). (B) Only tramadol: marked decrease of the positive reaction of the stain due to decreased body size of glial cells and shortening of their processes. (C) Tramadol + citicoline 50 mg/kg: mild amelioration of positive reaction of the stain. (D) Tramadol + citicoline 200 mg/kg: marked increase of positive reaction of the stain. Notice the increase in glial cell body size and length of processes.

dence for increased systemic oxidative stress following repeated tramadol administration. There was a significant increase in the lipid peroxidation end product MDA. The latter is the most widely used marker for lipid peroxidation and an increase in MDA is the evidence mostly cited to indicate the increase in the generation of free radicals in human disease. MDA derived from the oxidative damage to the molecular target, namely, polyunsaturated fatty acids [36, 37]. Increments in serum and plasma levels of products of lipid peroxidation, such as MDA or hydroperoxides indicative of systemic oxidative
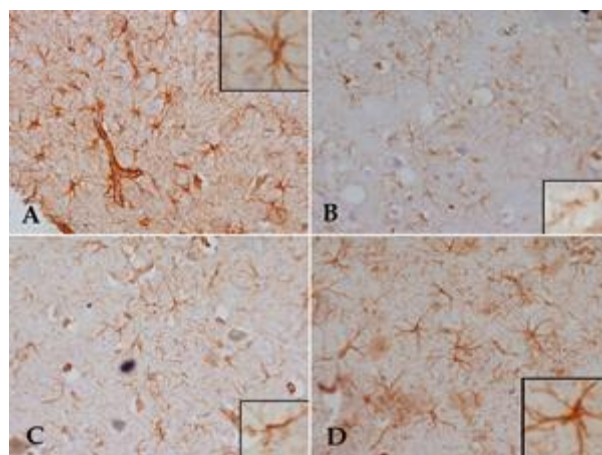
stress have been found in several disease processes including diabetes mellitus [38], liver disease [39], ischemic heart disease [40], Parkinson's disease [41], and Alzheimer's disease [42]. We also found that GSH, an important intracellular antioxidant and a scavenger of free radicals, was decreased in the serum of tramadol-treated rats, most probably due to its consumption by an increase in reactive free radicals. Other researchers reported a significant increase in MDA in the serum, brain, lung, and testicular tissue of rats following repeated administration of 40–50 mg/kg of tramadol [23, 43]. There were also significantly decreased GSH levels and reduced superoxide dismutase and catalase activities in the serum after tramadol treatment [23].

The present results also indicate markedly raised nitric oxide concentrations in the serum after tramadol. The gaseous molecule nitric oxide (NO) is

TABLE 4. Immunomorphometric measurements of GFAP in the brain of rats treated with saline, tramadol, or tramadol + citicoline

Group	GFAP (area%)
Saline	4.87 ± 0.31
Tramadol 30 mg/kg	0.88 ± 0.4[*]
Tramadol + citicoline 50 mg/kg	2.228 ± 0.596[*+] (153.4%)
Tramadol + citicoline 200 mg/kg	3.353 ± 0.254[*+] (281%)

Note: *, $p < 0.05$ vs. corresponding saline-treated group; +, $p < 0.05$ vs. tramadol only group. The percent change from the tramadol group is shown in parentheses.

generated from the amino acid L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase yielding nitric oxide and L-citrulline. There exist two constitutive nitric oxide synthase (NOS) isoforms, a neuronal one (nNOS, NOS-1) and an endothelial isoform (eNOS, NOS-3). In addition, there is an inducible isoform (iNOS, NOS-2), which is expressed in many cell types in pathological states in response to inflammatory stimuli, lipopolysaccharide endotoxin, or other agents [44]. Nitric oxide acts as a signaling messenger and under physiological conditions, the constitutively produced low levels of nitric oxide are important in intracellular signaling and in the regulation of basal vascular tone. In contrast, excess production of iNOS-derived nitric oxide by cells such as activated macrophages can cause tissue damage and organ dysfunction [45]. This occurs through the formation of more reactive nitrogen species, including nitrogen dioxide (formed by the reaction of nitric oxide with molecular oxygen) or peroxynitrite (formed by the reaction of nitric oxide and superoxide). These species are capable of oxidizing free thiols in the cytosol and amine, and causing thiol nitrosation and lipid peroxidation, leading to inhibition of mitochondrial enzymes, mitochondrial impairment, and cellular energy failure [46].

The present study also provides evidence for markedly decreased PON-1 activity in the serum by tramadol which is in agreement with our previous observations in which tramadol given at 5, 10, and 20 mg/kg resulted in a dose-dependent inhibition of serum PON-1 activity [47]. The PON-1 enzyme is known for its ability to hydrolyze the active metabolites, i.e., "oxons" of some organophosphate insecticides [48, 49]. The enzyme is also of interest for its role in atherogenesis, liver disease, and neurological disorders by virtue of its antioxidant and anti-inflammatory actions [49, 50]. It has been shown that the enzyme is able to reduce oxidative stress in the serum and macrophages, suppresses lipopolysaccharide- and interferon-γ-induced production of reactive oxygen species, tumor necrosis factor-α, and interleukin-6 from macrophages [51]. PON-1 activity is subject to modulation by dietary factors such as grape seed polyphenols, and drugs including aspirin and lipoic acid which increase the enzyme activity [52–54]. The enzyme is inactivated by organophosphate insecticides and by oxidative stress [55, 56]. In this study, the activity of the enzyme is reduced in the serum of tramadol-treated rats, which is likely to contribute, at least in part, to the observed increase in systemic oxidative stress. We also have shown that in tramadol-treated rats, the administration of citicoline was able to reverse the inhibition in PON-1 activity in a dose-dependent manner, thereby suggesting a potential benefit for citicoline in neurological or other disease processes where PON-1 activity is decreased.

We used the diphenylamine assay and the micronucleus test for the determination of DNA fragmentation in rats treated with tramadol. The in vitro micronucleus assay is widely used for the detection of chromosomal damage due to chemicals. Following chromosomal damage in mitotic cells, fragments or whole chromosomes tend to lag behind in the anaphase stage of cell division. These fragments or chromosomes are not incorporated in the nuclei of daughter cells and appear in the cytoplasm as single or multiple micronuclei [35]. Our results demonstrate that tramadol induced significant increases in the number of MnPCE in the bone marrow by 876.3%, compared to the saline control group. Quantification of bone marrow DNA fragmentation in the diphenylamine assay confirmed these observations where a 192.8% increase in DNA fragmentation was detected in tramadol-treated rats. These data suggest a muta-
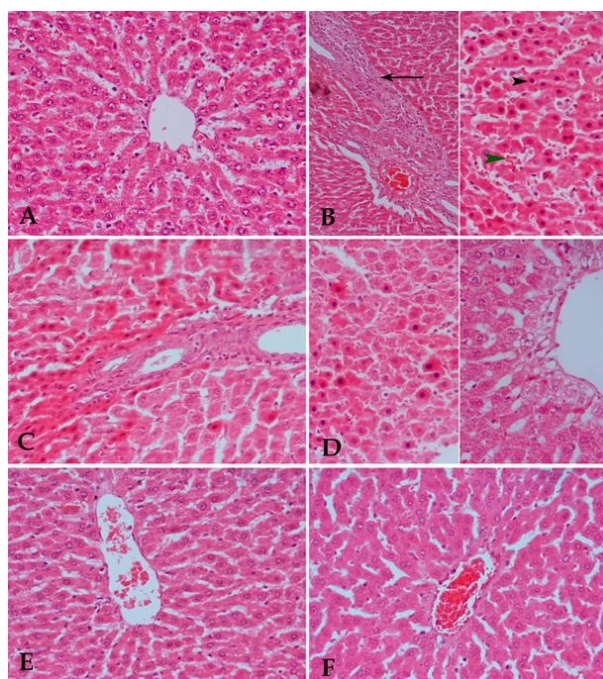
FIGURE 3. Representative photomicrographs of liver tissue sections after various treatments. (A) Saline: normal structure of hepatic cords and hepatocytes. (B) Only tramadol: it shows, in the left side of the figure, massive fibrosis extending in between the cells with inflammatory cells (arrow), and the right side shows many apoptotic hepatocytes with strong acidophilic cytoplasm and small dark nucleus (black arrowhead). Dilated and congested blood sinusoids are also observed (green arrowhead). (C) Tramadol + citicoline 50 mg/kg: fibrosis is still observed and with slight reduction of damaged cells. (D) Tramadol + citicoline 100 mg/kg: it shows, in the left side of figure, marked reduction of damaged cells, while the right side shows slight vacuolar degeneration of some cells around the central vein with no fibrosis or inflammatory cells. (E) Tramadol + citicoline 200 mg/kg: mild dilatation and congestion of main blood vessels and normal hepatocytes. (F) Only citicoline 200 mg/kg: quite normal liver tissue structure. H&E staining.

FIGURE 4. Representative photomicrographs of kidney tissue sections after various treatments. (A) Saline: normal structure of glomeruli and tubules. (B) Only tramadol: marked vacuolar degeneration of tubular lining epithelium and edema of glomeruli. (C) Tramadol + citicoline 50 mg/kg: vacuolar degeneration of cells is still noticed with capillary dilatation and congestion. (D) Tramadol + citicoline 100 mg/kg: marked reduction of vacuolar degeneration and signs of edema. (E) Tramadol + citicoline 200 mg/kg: normal shaped tubules except for a few degenerated cells. (F) Only citicoline 200 mg/kg: quite normal kidney tissue structure. H&E staining.

genic effect for tramadol at the dose used in the study. These changes were significantly decreased, but not prevented, by citicoline, suggesting a protective effect.
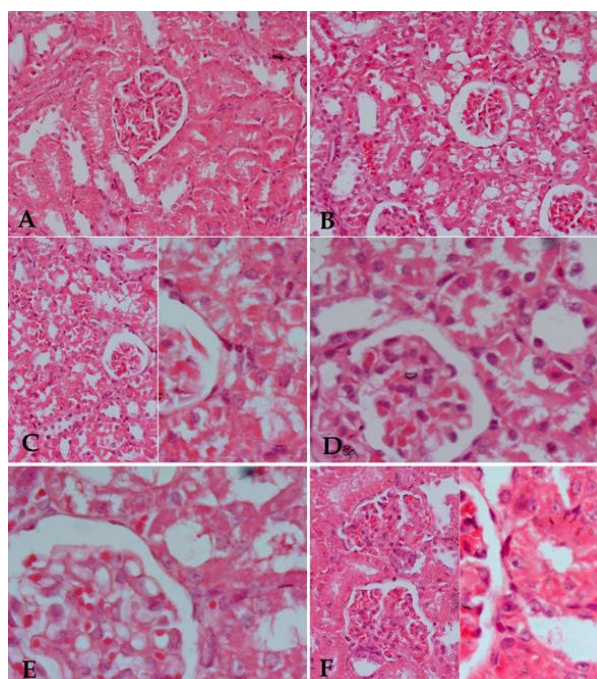
The present study clearly indicates that the repeated administration of tramadol at the dose of 30 mg/kg was capable of inducing neuronal changes in the cerebral cortex of treated rats with signs of neuronal degeneration (dark nuclei and acidophilic cytoplasm). Other researchers reported the presence of darkly stained pyramidal cells with pyknotic nuclei and perineuronal haloes, shrunken neurons, and cytoplasmic vacuolization in cerebral cortex of rats treated with 50 mg/kg of tramadol for 4 weeks. There was also increased Bax immunoreactivity,

suggesting increased apoptosis [43]. We, in addition, showed that tramadol led to decreased GFAP immunostaining in cerebral cortex. GFAP is an intermediate filament protein expressed in cell bodies and processes of astrocytes and is a major constituent of their cytoskeleton. Increased GFAP expression is considered a specific marker of astrocyte activation, a process called astrogliosis seen in ischemic/hypoxic, toxic, and inflammatory (and neurodegenerative) conditions [57, 58]. Astrocytes also perform a number of important functions such as the production of extracellular matrix proteins and adhesion molecules, neurite promoting factors, regulation of pH and ion concentration, and uptake of glucose and glutamate [59]. Our data suggest decreased GFAP in astrocytes by tramadol together with decreased body size and shortening of their processes, which is likely to have important adverse effects on neuronal functions. We also showed that treatment with citicoline was able to prevent the toxic effects of tramadol on neurons and astrocytic cells with marked increase of GFAP immunostaining together with an increase in glial cell body size and length of astrocyte processes.

Our study also demonstrated adverse effects for tramadol on the liver and kidney evidenced by the presence of apoptotic hepatocytes, fibrosis, and inflammatory cell infiltration in the liver, as well as vacuolar degeneration of the tubular lining epithelium and glomerular edema in the kidney. In their study, Hafez et al. [60] found foci of hepatic necrosis and mononuclear inflammatory cell aggregation in rats given 50 mg/kg of tramadol daily for 2 weeks. Rats treated with the drug at 40 mg/kg for 20 days exhibited severe hydropic degeneration in the liver and atrophied glomeruli and degenerated renal tubules [23]. In the present study, the pathological changes caused by tramadol in the liver and kidney were all ameliorated by citicoline.

In this study, tramadol is given at a dose of 30 mg/kg/day which is approximate to the human dose of 300 mg/day. Tramadol is usually administered at a dose of 50–200 mg/day, the upper limit is 400–600 mg/day [61, 62]. There are reports, however, indicating the use of doses up to 675 mg/day [14], 800–2000 mg [63], 1500 mg per day [64], and 2500 mg [65], which, when converted to that of rats, are much higher than that employed in our study. The dose used in the present study is thus relevant to the doses used by abusers of tramadol, and it is thus likely that

similar pathological changes could be occurring in those subjects who take exceedingly high doses of the drug.

The findings in the present study indicate that citicoline was able to alleviate the biochemical and histopathological alterations induced by tramadol. Citicoline has been shown to decrease brain permeability that occurs in endotoxemia and after intracerebral hemorrhage. Citicoline showed neuroprotective effects in experimental models of closed head injury, decreasing brain edema and blood brain barrier permeability. The drug increased superoxide dismutase activity and GSH content, and decreased lipid peroxidation (MDA) and neuronal cell death [66]. It also showed benefit slowing the progression of the disease when given in combination with rivastigmine in subjects suffering from Alzheimer's disease and mixed dementia [67].

In conclusion, tramadol given to rats at the dose of 30 mg/kg for two weeks induced systemic oxidative stress and DNA fragmentation while cellular damage was encountered in the brain, liver and kidney tissues. Citicoline, a choline donor, in use for treating brain disorders, was able to ameliorate oxidative stress and DNA fragmentation, and prevented the tramadol-induced pathological changes. These effects of citicoline are likely to be mediated by counteracting tramadol-induced oxidative stress. Our data, therefore, suggest a beneficial role for citicoline in the management of patients who abuse or who are intoxicated with tramadol.

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