

# Neurotoxicology

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## Full Length Article

# Does nicotine impact tramadol abuse? Insights from neurochemical and neurobehavioral changes in mice

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## ABSTRACT

Nicotine and tramadol concomitant drug dependence pose increasing social, economic as well as public threats. Accordingly, the present study investigated neurochemical, neurobehavioral and neuropathological changes in the brain subsequent to the interaction of nicotine and tramadol. To this end, tramadol (20 mg/kg, i.p.) and nicotine (0.25 mg/kg, i.p.) were administered to male albino mice once daily for 30 days. Consequent to microglial activation, nicotine exacerbated oxidative/nitrosative stress induced by tramadol as manifest by the step-up in thiobarbituric acid reactive substances and nitric oxide subsequent to the enhanced levels of neuronal and inducible nitric oxide synthases; paralleled by decreased non-protein sulphydryls. Increased oxidative stress by tramadol and/or nicotine sequentially augmented nuclear factor kappa B and the proinflammatory cytokine tumor necrosis factor  $\alpha$  with the induction of apoptosis evident by the increased caspase-3 immunoreactivity. However, paradoxical to the boosted inflammation and apoptosis, heightened DA levels in the cortex parallel along with increased tyrosine hydroxylase in midbrain were apparent. Concomitant administration of tramadol and nicotine impaired spatial navigation in the Morris Water Maze test coupled with enhanced levels of acetyl- and butyryl cholinesterases. However, tramadol in association with nicotine improved social interaction while decreasing anxiety and aggression linked to chronic administration of nicotine, effects manifested by increased levels of serotonin and GABA. These results provide evidence that co-administration of tramadol and nicotine may enhance reward and dependence while reducing anxiety and aggression linked to nicotine administration. However, such combination exacerbated neurotoxic effects and elicited negative effects regarding learning and memory.

## 1. Introduction

Drug addiction, including polydrug use, can be defined as a maladaptive behavior which is characterized by "loss of control" over drug use (Balconi et al., 2014). The diversity and abuse of prescription drugs, particularly opioids is a major worldwide tendency (Simonsen et al., 2015). Nonetheless, concomitant drug dependence is not only limited to prescription medications but also encompasses nicotine and opioids. In this context, some epidemiological studies revealed that tobacco smoking and illicit drug use are frequently a co-occurring behavior

(Frosch et al., 2000; Elkader et al., 2009; Epstein et al., 2010).

Tramadol, a centrally acting synthetic opioid, that possesses anti-nociceptive and analgesic effects (Raffa, 2008) has emerged rapidly as a drug of abuse in the past few years (Shalaby et al., 2015). It has been postulated that the pharmacological effects of tramadol may be mediated by both opioid ( $\mu$ -receptor agonist) and non-opioid (nor-epinephrine and serotonin reuptake inhibition) mechanisms (Raffa et al., 1992; Liu et al., 1999; Nossaman et al., 2010), with the involvement of both in development of drug addiction (Zhuo et al., 2012). Noteworthy, tramadol metabolite, o-desmethyl tramadol (M1), is also

**Abbreviations:** AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; GABA, gamma aminobutyric acid; GLU, Glutamate; GSH, reduced glutathione; iNOS, Inducible nitric oxide synthase; LLE, liquid liquid extraction; M<sub>1</sub>, O-desmethyl tramadol; MWM, morris water maze; NF $\kappa$ B, nuclear factor kappa B; NMDA, n-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NPSH, non protein sulphydryl; OFT, open field test; RNS, reactive nitrosative species; ROS, reactive oxygen species; SI, social interaction; TBARS, thiobarbituric acid reactive substances; TH, tyrosine hydroxylase; TNF- $\alpha$ , tumor necrosis factor alpha; VTA, ventral tegmental area

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active as  $\mu$ -receptor agonist, such that the long-term use of tramadol can induce tolerance, physical dependence and withdrawal symptoms (Abdel-Zaher et al., 2011). Addicts may revert to tramadol combinations with other substances of abuse either to increase euphoria, achieve more favorable effects, act as a substitute or avoid/relieve the opioid-related withdrawal symptoms (Gibson, 1996; Liu et al., 1999); however, these practices have augmented the number of deaths in recent years (Sheikholeslami et al., 2016).

Tobacco consumption is one of the main public health problems worldwide and represents a leading cause of preventable deaths in most developed countries (Dwoskin et al., 2009; Berrendero et al., 2010). Nicotine, the reinforcing addictive component of tobacco, is the major arbitrator that maintains the smoking habit (Shoaib, 2006). Like other addictive drugs, nicotine displays the two important characteristics to establishing and maintaining addiction; first, by eliciting pleasant or rewarding effects in the brain which reinforce self-administration behavior; and secondly, appearance of withdrawal syndrome following chronic exposure to nicotine which favors continued intake (Lewis et al., 2007).

Neurochemical studies revealed that nicotine and opioids modulate each other effect (Skurtveit et al., 2010) and nicotine induced DA release via activation of  $\mu$  opioid receptors (Tanda and Di Chiara, 1998). Moreover, experimental studies highlighted the existence for a mechanistic overlap between opiates and nicotine within dopamine (DA) reward pathway (Britt and McGehee, 2008; Berrendero et al., 2010). In light of these considerations, the aim of this work was to investigate the possible modulatory effects of nicotine on tramadol-induced neurobehavioral and neuropathological changes in the brain. In addition, an attempt was undertaken to clarify the possible neurochemical alterations in neurotransmitters, inflammation and oxidative/nitrosative stress.

## 2. Material and methods

### 2.1. Animals

Adult male Swiss albino mice weighing 25–30 g were purchased from the animal house colony of the national research center (NRC, Giza, Egypt). Mice were allowed one week acclimatization period and were housed in groups at constant temperature ( $23 \pm 2^\circ\text{C}$ ), humidity ( $60 \pm 10\%$ ) and a light/dark (12/12 h) cycle with lights on at 5:00 am. They were allowed free access to food and water throughout the experimental period. All behavioral experiments were carried out in separate and isolated laboratories. The study was performed in compliance with the procedures and policies approved by the Research Ethics Committee of Faculty of Pharmacy, Cairo University (Cairo, Egypt; PT 1452) in compliance with the Guide for the Care and Use of Laboratory Animals (ILAR, 1996).

### 2.2. Drugs and chemicals

Tramadol hydrochloride was obtained from Memphis Company (Cairo, Egypt); nicotine solution was purchased from Sigma-Aldrich (MO, USA). All other chemicals and reagents used for biochemical estimations were of analytical grade.

### 2.3. Drug treatment schedule

Mice were divided in to 4 groups and treated as follows: (i) group I served as control and received the vehicle; (ii) animals in group II were given tramadol (20 mg / kg, i.p); (iii) group III received nicotine (0.25 mg/kg, i.p); and (iv) group IV was given tramadol and nicotine at the same doses mentioned earlier. All agents were dissolved in saline and administrated once daily for thirty days. All animals were subjected to behavioral experiments that were performed at the same time-period of the day (09:00 am -12:00 pm). Behavioral tests were carried out

throughout the last week of the experiment while body weight was taken on weekly basis. Following the exclusion of dead animals in each group, the experimental groups I and III consisted of (6–8 animals) whereas groups II and IV included (12–14 animals). Thirty minutes following last tramadol injection in groups II and IV, (4–6 mice) were euthanized and the brains were isolated for detection of tramadol in brain. Twenty-four hours following the last treatment, (6–8 mice each group) were euthanized with cervical dislocation and brains were isolated. Cortices were dissected over ice for neurotransmitters and biochemical analysis. Part of cortical tissue was homogenized in 75% methanol (HPLC grade, Sigma-Aldrich) and cortical monoamines were measured. Derivatization of dried cortical homogenate was carried out for determination of GABA and glutamate. The rest of cortical tissue was homogenized in phosphate buffer saline (pH 7.4) for estimation of other mediators in cortices. Samples were stored at  $-80^\circ\text{C}$  till analysis.

### 2.4. Detection of tramadol in brain

Thirty minutes following the last injection, mice were euthanized with cervical dislocation and brains were isolated and homogenized in Tris – HCl buffer. Extraction of tramadol from brain homogenates was performed according to the method described by Tao et al. (2001). Briefly, methanol was used for protein precipitation and methyl tertiary butyl ether (MTBE) was utilized for liquid-liquid extraction (LLE). Subsequently, the MTBE layer was evaporated under nitrogen flow and the residue was reconstituted with 50  $\mu\text{l}$  of methanol (HPLC grade). A sample volume of 3  $\mu\text{l}$  was injected in GC-MS (Agilent 6890 Network capillary gas chromatograph equipped with a mass selective detector Agilent 5973 Network; CA, USA) using dextromethorphan as internal standard (I.S.).

### 2.5. Biochemical measurements

#### 2.5.1. Determination of cortical TBARS, NPSHs and NO contents

Thiobarbituric acid reactive substances (TBARS) content was measured according to the method described by Mihara and Uchiyama (1978). In brief, thiobarbituric acid in o-phosphoric acid was added to the homogenate and boiled for 20 min, cooled and centrifuged at 1000 g for 10 min then the resultant complex was read at 532 nm. Non protein sulphydryls (NPSHs) content was measured according to the method of Ellman (1959) and modified by Beutler et al. (1963). After precipitation of protein, Ellman's reagent (5, 5'-dithiobi-2-nitrobenzoic acid) was reduced by sulphydryl (SH) group to form 2-nitro-5-mercaptopbenzoic acid that has an intense yellow color and can be determined colorimetrically at 412 nm. Nitric oxide (NO) was determined using Griess reagent according to the method described by Miranda et al. (2001), the absorbance was read at 540 nm.

#### 2.5.2. Determination of cortical TNF- $\alpha$ , NFkB, iNOS and nNOS

ELISA kits for tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ; CSB-E04741), nuclear factor kappa B (NFkB; CSB-E12108) and inducible nitric oxide synthase (iNOS; CSB-E08326) were purchased from Cusabio (Wuhan, PRC) whereas neuronal nitric oxide synthase (nNOS; MBS753956) was obtained from MyBioSource (CA, USA) and used according to the manufacturers' instructions.

#### 2.5.3. Determination of cortical monoamines and amino acids

Estimation of dopamine (DA), serotonin (5 HT) and norepinephrine (NE) was carried out according to the method of Pagel et al. (2000) using HPLC (Perkin-Elmer, MA, USA). Briefly, homogenized samples were extracted from trace elements and lipids by solid phase extraction. Each sample (40  $\mu\text{l}$ ) was filtered (0.25  $\mu\text{m}$ , Millipore, MA, USA) then injected into an AQUA column (C18, 150  $\times$  4.6 mm, 5  $\mu\text{m}$ , Phenomenex, CA, USA), using mobile phase (methanol: acetonitrile; 97:3 v), flow rate 1.5 ml/min, and peaks were detected at 270 nm. Moreover, glutamate (GLU) and gamma amino butyric acid (GABA) were

estimated according to the method of Heinrikson and Meredith (1984), using the HPLC system (Perkin-Elmer). The reconstituted cortical residues (methanol: 1 M sodium acetate: triethylamine; 2:2:1 v) were redried under vacuum. Using the precolumn isothiocyanate derivatization technique, samples were subjected to a 20 min derivatization performed using a methanol: triethylamine: double-distilled deionized water: phenylisothiocyanate (7:1:1:1 v), redried under vacuum, then reconstituted with sample diluents (acetonitrile: 5 mM phosphate buffer; 5:95 v; pH = 7.2). Sonicated and filtered (0.45 µm; Millipore) samples were run on a Pico-Tag C18 (300 mm × 3.9 mm, i.d., Waters, CT, USA) and a binary gradient of Eluents 1 and 2 (Waters) were used. Column temperature (46 ± 1 °C) and a constant flow rate (1 ml/min) were sustained throughout the experiment. A sample volume of 20 µl was injected and the corresponding peaks were detected at 254 nm.

#### 2.5.4. Determination of cortical acetyl- / butyrylcholinesterases

The procedure used for acetylcholinesterase (AChE) was a modification of the method of Ellman et al. (1961) as described by Gorun et al. (1978). The principle of the method is the measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color was read immediately at 412 nm. Butyrylcholinesterase (BuChE) activity in cortical supernatant was determined using butyrylcholinesterase diagnostic kit purchased from Biodiagnostic (Cairo, Egypt) according to the method of Knedel and Bottger (1967).

### 2.6. Behavioral tests

#### 2.6.1. Open field test (OFT)

The open field test was performed according to the method described by Bailey and Crawley (2009). The OF is a square wooden arena (40 cm x 40 cm x 20 cm high) with red walls and white smooth polished floor that is divided into a grid of equally sized areas by lines drawn on the chamber floor for visual scoring of activity by the experimenter. The following parameters were recorded during the 5 min observation period: (i) thigmotaxis time (time spent by the animal exploring the periphery of the arena, usually in contact with the walls and corners); (ii) central square frequency: (frequency with which the mice crossed with all four paws into the central square area); (iii) ambulation frequency: number of squares crossed by the animal; (iv) rearing frequency: number of times the animal stood stretched on its hind limbs with or without forelimb support and (v) grooming frequency: number of face scratching, washing with the hind limbs and licking of the forelimbs.

#### 2.6.2. Social interaction test (SI)

Test was performed according to the method described by Niijima-Yaoita et al. (2013). The apparatus for the SI test was made of a clear polycarbonate (25.7 cm x 30.8 cm x 18.0 cm high). After habituation for 30 min, the mouse to be tested was randomly assigned to an unfamiliar partner (visitor mouse) of the same strain, age and sex. The pair of unfamiliar mice was placed in the apparatus for 10 min and the total amount of time spent in active SI, such as sniffing, grooming, following and mounting as well as crawling over or under the visitor mouse in direct contact was recorded.

#### 2.6.3. Defensive aggression test

Test was performed according to the method described by Johansson et al. (2000). For the defensive aggression test, the mouse was lifted by its tail and placed in a Plexiglas cage (60 × 31 × 41 cm high) and allowed to habituate for 30 s. The reaction of each mouse to four different stimuli was then assessed. (i) A wooden rod was slowly moved to approach and touch the mouse's snout (0: no response or sniffs at the rod; 1: intermittently bites or attacks the rod and/or adopts a defensive upright posture; 2: continuously bites/attacks the rod). (ii) Startle to an air puff (air blown from a 50-ml syringe) at the back (0: no response or some movement; 1: jumping response; 2: exaggerated

jumping response). (iii) Poking with wooden rod at the flanks (0: no response or sniffing at the rod; 1: defensive upright posture; 2: defensive upright posture together with biting/attack). (iv) Capturing with a gloved hand (0: very easy to capture; 1: easy to capture but some resistance and/or prolonged vocalization; 2: difficult to capture because of escape; 3: difficult to capture because of attacking or biting; 4: very difficult to capture because of continuous violent attacks/bites). The averages of the total scores (maximum total score 10) from each individual stimuli test was used in the statistical calculations.

#### 2.6.4. Morris Water Maze test (MWM)

**Trail Test:** Individual animals were tested in a spatial version of Morris Water Maze test according to the method described by Bromley-Brits et al. (2011). The maze consists of a large circular pool with a diameter of 150 cm and a depth of 50 cm of water maintained at room temperature. A platform (10 cm diameter) was located in the pool and concealed below the surface of the water (i.e., ~ 1 cm). The platform was rendered invisible by making the water opaque using powdered milk. The water maze task was carried out in the last week of the experiment. Mice received five consecutive daily training trials in the following 5 days, with each trial exhibiting a cut-off time of 60 s and a trial interval of approximately 30 s. If the mouse failed to reach the escape platform within the maximally allowed time of 60 s, it was guided with the help of the experimenter and allowed to remain on the platform for 20 s. The time to reach the platform (escape latency in seconds) was measured.

**Memory consolidation test (probe trial):** The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe trial, the mouse was placed into the pool as in the training trial, except that the hidden platform was removed from the pool. The total time spent in target quadrant in a period of 60 s was recorded (Bromley-Brits et al., 2011).

### 2.7. Histopathological examination

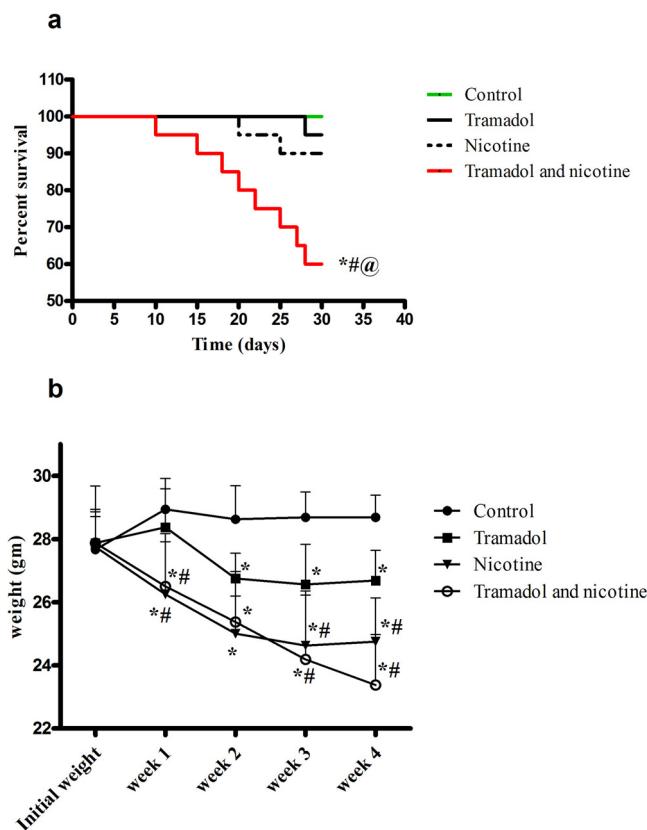
The brain of each mouse was carefully dissected out and fixed in 10% buffered neutral formalin. The fixed brain specimens were routinely dehydrated by graded series of alcohol, cleared in xylol and finally embedded in paraffin. Paraffin blocks were serially sectioned at 4 µm thickness and stained with haematoxylin and eosin stains (H&E) (Bancroft and Gamble, 2008). The obtained sections were collected on glass slides, and subjected to histopathological examination using the electric light microscope Olympus BH2 (Tokyo, Japan).

### 2.8. Immunohistochemical examination of CD68, Casp-3 and TH

For recognition of CD68, caspase-(Casp)-3 and tyrosine hydroxylase (TH) reactive cells, immunohistochemical procedures were carried out on 4 µm paraffin sections of brain tissue of control and different treated groups using avidin-biotin peroxidase according to the method described by Hsu et al. (1981). Briefly, paraffin sections were deparaffinized in toluene, rehydrated in ethanol, and then incubated with H<sub>2</sub>O<sub>2</sub> for blocking the endogenous peroxidase activity. The sections were incubated with a monoclonal antibody for CD68, casp-3 and TH (Dako Corp, CA, USA). The immune reactive cells for each marker were visualized using the chromagen 3, 3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, MO, USA). The number of apoptotic cells (Casp-3 dependent) was determined by counting the number of positive cells in 5 randomly chosen microscopic fields at 40x objective magnification, the area for each microscopic field is 18.8913Sqm.

### 2.9. Statistical analysis

All values are presented as means ± standard deviation of the means (SD). Comparison between groups was carried out using the non-parametric one-way analysis of variance (ANOVA) followed by Tukey-



**Fig. 1. (a)**: Kaplan-Meier survival curve ( $N = 20$ ) showing higher survival with tramadol and nicotine alone compared to the group receiving both drugs. No significant change in survival was observed between tramadol and nicotine groups. P-values were calculated with log-rank (Mantel-Cox) test. **(b)**: Tramadol administration induced a significant decrease in BW from the 2<sup>nd</sup> week of the test. Mice subjected to nicotine either alone or in combination with tramadol showed a significant decrease in BW from the 1<sup>st</sup> week of the experiment. Data are expressed as mean of (8) experiments  $\pm$  SD. Statistical analysis were carried out by two way ANOVA followed by Bonferroni post test. \*: significant from control, #: significant from tramadol, @: significant from nicotine at  $P < 0.05$ .

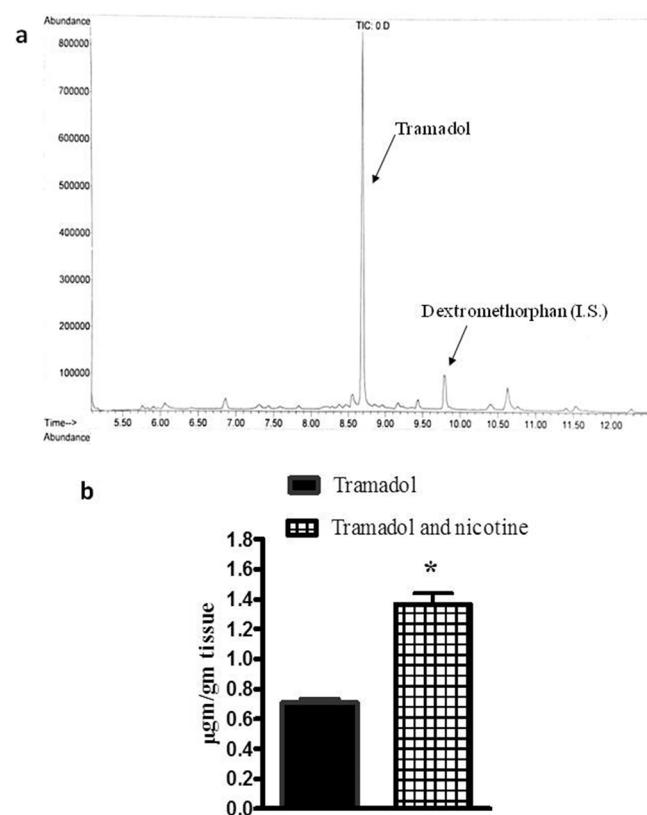
Kramer Multiple Comparison Test for all the parameters. For the body weight change and MWM, two-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparison Test was used. Difference was considered significant when  $P < 0.05$ . Survival analysis was carried out using Kaplan-Meier plots and log rank (Mantel-Cox) tests. Graphpad Prism® 5.00 for Windows Software (CA, USA) was used to carry out these statistical tests and graphical presentation.

### 3. Results

#### 3.1. Survival analysis and body weight change

Unlike the control group that displayed 100% survival, groups in the tramadol, nicotine or their combination groups showed progressive decline in survival. Tramadol alone had significantly higher survival compared to group receiving tramadol and nicotine (95% vs 60% survival;  $p = 0.0068$ ). In addition, simultaneous administration of nicotine with tramadol significantly lowered survival compared to nicotine per se (60% vs 90% survival;  $p = 0.0286$ ). No significant change in survival was observed between tramadol and nicotine groups (Fig. 1a).

Injection of tramadol (20 mg/kg, i.p.) produced a significant decrease in body weight (BW) starting the 2<sup>nd</sup> week and through the end of the 4<sup>th</sup> week of drug administration compared to control (Fig. 1b). By the same token, nicotine induced a noticeable decline starting from the



**Fig. 2. (a)**: Representative chromatogram of an extract of a brain sample collected from a mouse receiving (20 mg/kg) of tramadol 30 min prior to sample collection. **(b)**: Animals receiving tramadol + nicotine displayed an increment in tramadol concentration in the brain versus those receiving tramadol alone. Data are expressed as mean of (4–6) experiments  $\pm$  SD. Statistical analyses were carried out by ANOVA followed by Tukey's Multiple Comparison Test. \*: significant from tramadol at  $P < 0.05$ .

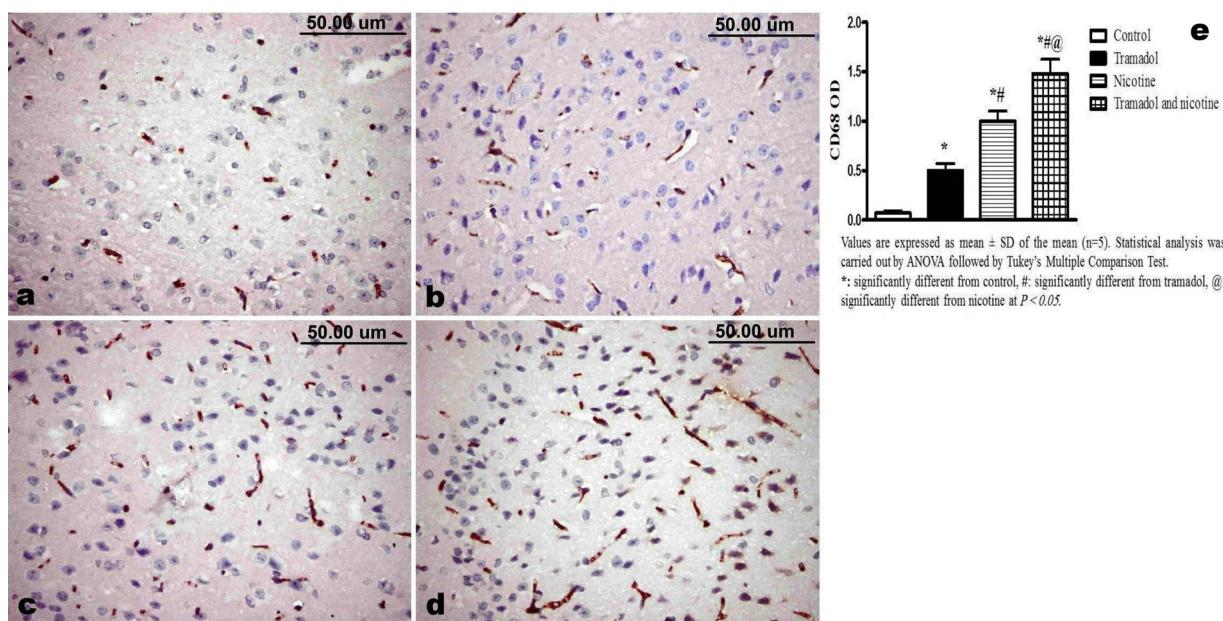
1<sup>st</sup> week compared to normal control mice, which by the end of the 4<sup>th</sup> week, showed a significant decrease (13.7%) compared to their control counterparts (Fig. 1b). As illustrated in (Fig. 1b), at the end of 4<sup>th</sup> week there was a significant drop in body weight by 18.5 and 12% compared to control and tramadol, respectively with nicotine and tramadol co-administration.

#### 3.2. Changes in tramadol concentration in mice brain induced by concomitant administration of nicotine with tramadol

Tramadol (20 mg/kg, i.p.) was rapidly absorbed with a concentration mounting to 0.71  $\mu$ g/g in the brain after 30 min (Fig. 2a). A significant boost in tramadol concentration was observed with the co-administration of nicotine (0.25 mg/kg, i.p.) and tramadol produced reaching 91%, compared to tramadol group (Fig. 2a, b).

#### 3.3. Alterations in CD68 immunoreactivity induced by tramadol, nicotine and their combined administration

CD68 immunoreactivity showed distinctive rod-like morphology of microglia in the examined cerebral cortices of control and all treated groups. The cerebral cortices of control mice revealed appearance of few scattered immunoreactive CD68 microglia (Fig. 3a); while both tramadol and nicotine administration showed increased immunoreactive microglia cells (Fig. 3b-c). The combined administration of tramadol and nicotine resulted in more activation of microglia cells denoted by marked increased expression of CD68 (Fig. 3d).



**Fig. 3.** Photomicrograph depicting CD68 immunohistochemical staining in the cerebral cortex. Brown color (positive) indicates specific immunostaining for CD68. Control mice showing few scattered CD68 immunopositive microglia cells (**a**). Increased immunoreactivity of CD68 among activated microglia cells after tramadol and nicotine administration (**b** and **c**). Marked increased activation of microglia expressing CD68 in mice subjected to tramadol and nicotine (**d**). Quantitative image for immunohistochemical staining expressed as optical densities (OD) across 10 different fields for each mouse section (**e**).

#### 3.4. Effect of nicotine on tramadol-induced alterations in cortical TBARS, NPSHs and NO contents in mice

Tramadol and nicotine given alone produced a buildup in TBARS (46.5 and 63%, respectively) and NO contents by (1.4 and 1.2 fold, respectively) accompanied by a decline NPSHs (20 and 30%, respectively) compared to control group (Fig. 4a-c). By the same token, nicotine given concurrently with tramadol elevated TBARS and NO levels by 76.8 and 180%, respectively, paralleled with a reduction in NPSHs level by 31.5% compared to control group. Interestingly, the increase in TBARS and NO was significant compared to each drug alone (Fig. 4a-c).

#### 3.5. Changes in nNOS and iNOS induced by tramadol either alone or in combination with nicotine

Tramadol as well as nicotine elevated nNOS (2.1 and 3.1 folds, respectively) as well as iNOS (2.1 and 6.3 fold, respectively) in cerebral cortex compared to control (Fig. 5c-d). On the other hand, administration of nicotine concurrently with tramadol augmented the elevation in concentrations of nNOS (4.3 fold) and iNOS (8.2 fold) compared to control group (Fig. 5c-d), with values significantly greater when compared to either tramadol or nicotine groups.

#### 3.6. Effect of nicotine on tramadol – induced alterations in cortical NF $\kappa$ B, TNF- $\alpha$ as well as Casp-3 immunoreactivity

The transcription factor (NF $\kappa$ B) and its downstream proinflammatory cytokine (TNF- $\alpha$ ) were raised by the single administration of tramadol (1 fold, each) or nicotine (3.2 and 2.3 folds respectively) in cerebral cortex compared to their control counterparts (Fig. 5a-b). The step-up in NF $\kappa$ B and TNF- $\alpha$  levels induced by nicotine was remarkable compared to tramadol group (Fig. 5a-b). Furthermore, co-administration of nicotine with tramadol induced significant increases in NF $\kappa$ B and TNF- $\alpha$  compared to tramadol and nicotine groups (Fig. 5a-b). Additionally, control mice showed negative expression of Casp-3 in cerebral cortex (Fig. 6a). Conversely, both tramadol and nicotine alone or in combination showed Casp-3 immunoreactive positive cells depicted by the intense brown staining (Figs. 6b-e). The number of

apoptotic cells (Casp-3 dependent) was determined by counting the number of positive cells in 5 randomly chosen microscopic fields at 40 x objective magnification (Fig. 6f).

#### 3.7. Changes in cortical DA level as well as midbrain TH immunoreactivity induced by tramadol either alone or in combination with nicotine

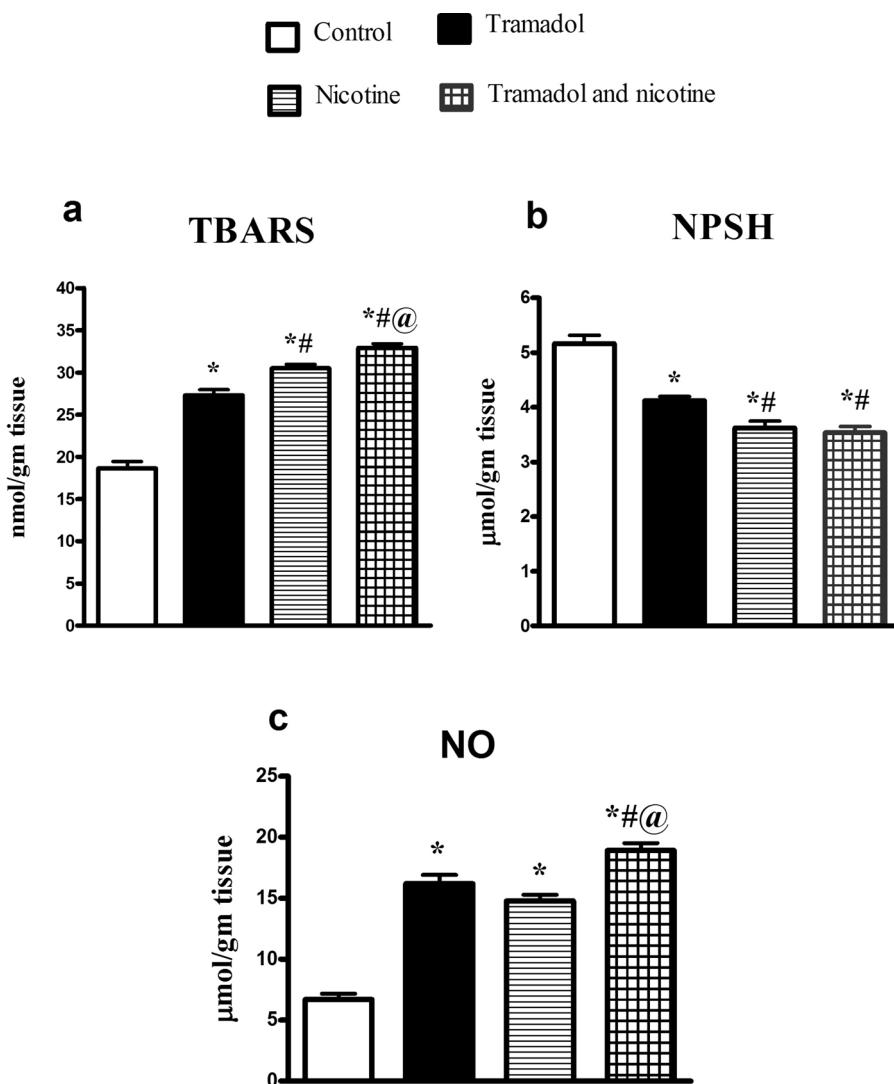
Following 24 h of the last injection, DA was significantly elevated in cerebral cortex of mice subjected to tramadol or nicotine by 65 and 33.6%, respectively compared to control group (Table 1). Moreover, mice given tramadol on top of nicotine showed increased DA level by 61 and 20.4%, respectively in cerebral cortex compared to control and nicotine groups. Nonetheless, the increase in DA level showed no significance compared to tramadol group (Table 1). Furthermore, control group showed minimal immunostaining for TH (Fig. 7a). Both of tramadol and nicotine alone or in combination induced more TH immunoreactive cells (Figs. 7b-d).

#### 3.8. Changes in cortical 5 HT and NE levels induced by tramadol either alone or in combination with nicotine

Mice treated with tramadol and nicotine showed a buildup in cortical NE (20.8 and 43.7%, respectively), however only those receiving tramadol displayed an increment in cortical 5 HT level (66.6%) compared to their control counterparts (Table 1). In addition, co-administration of nicotine concurrently with tramadol increased 5 HT by 44 and 25.6% compared to nicotine and control groups, respectively. Furthermore, mice given tramadol and nicotine displayed a significant increase in NE level (16.6%) compared to control group (Table 1).

#### 3.9. Changes in cortical glutamate and GABA levels induced by tramadol either alone or in combination with nicotine

Single administration of tramadol as well as nicotine elevated cortical glutamate by 24.3 and 29.3% compared to their control counterparts (Table 1). Conversely, only tramadol induced an increment in GABA (69.3%), the latter being reduced by nicotine (23%) compared to control group (Table 1). Furthermore, the increase in glutamate level



**Fig. 4.** Mice given tramadol or nicotine in addition to tramadol showed increased TBARS (a) lowered NPSH (b) in addition to elevated nitric oxide (c) levels. Data are expressed as mean of (6–8) experiments  $\pm$  SD. Statistical analyses were carried out by ANOVA followed by Tukey's Multiple Comparison Test. \*: significant from control, #: significant from tramadol at  $P < 0.05$ .

(79.8%) induced by the co-administration of tramadol and nicotine was significant compared to control, as well as to that produced by tramadol or nicotine alone. Meanwhile, there was a significant increase in GABA level compared to control and nicotine groups (Table 1).

### 3.10. Effect of tramadol either alone or in combination with nicotine on acetyl- and butyrylcholinesterases in cerebral cortex

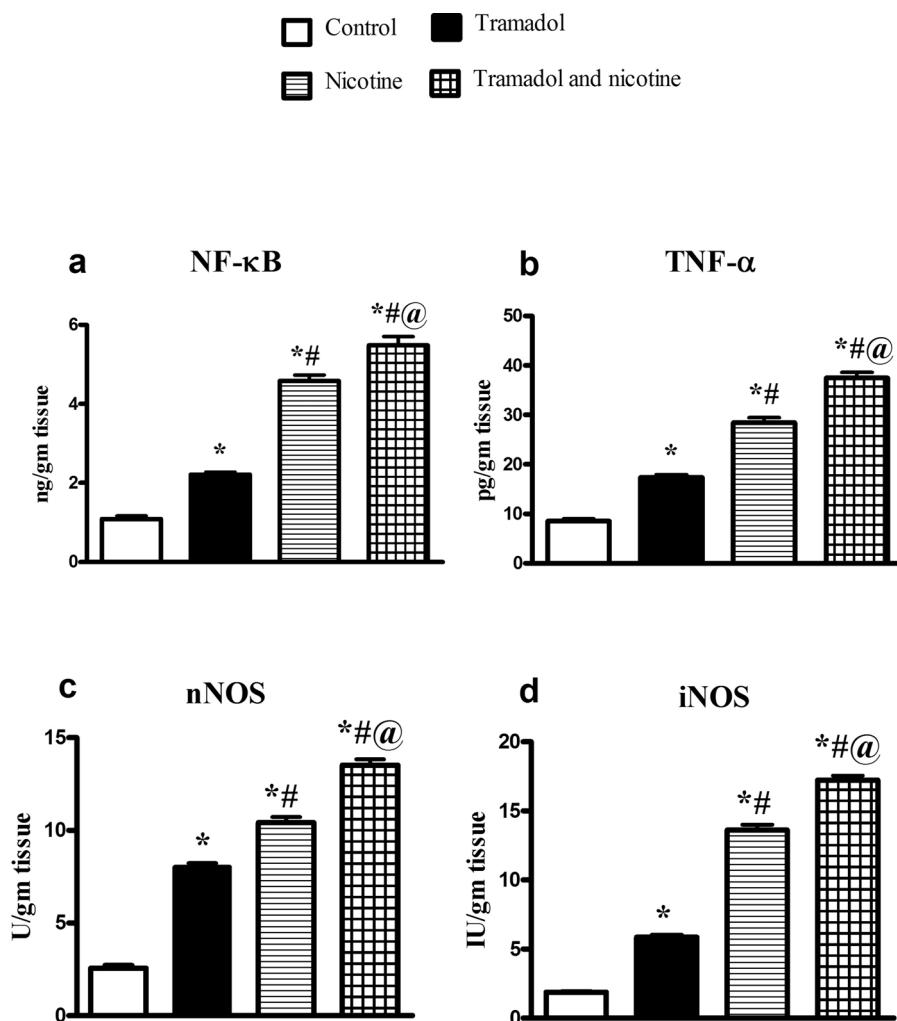
Mice treated with tramadol showed no change in acetyl cholinesterase concentration and butyrylcholinesterase activity in cerebral cortex (Fig. 8a–b). Nicotine produced a significant increase in cortical acetylcholinesterase by 51.2 and 35.4% compared to control and tramadol groups, respectively. However, nicotine had no effect on butyrylcholinesterase activity (Fig. 8a–b). Furthermore, nicotine administered concurrently with tramadol induced elevation in acetylcholinesterase concentration and butyrylcholinesterase activity compared to control and tramadol groups (Fig. 8a–b). However, this increase was not significant compared to nicotine group.

### 3.11. Alterations in spatial memory in the Morris Water Maze test consequent to tramadol administration either alone or in combination with nicotine

The escape latencies to reach hidden platform decreased gradually in control group, indicating good learning performance (Fig. 9a). In opposition, mice subjected to tramadol and nicotine either alone or in combination showed a disturbed performance in MWM with different extents, with tramadol showing the least disturbed performance (Fig. 9a). The escape latency was significantly increased in case of nicotine treated mice compared to tramadol and control groups, indicating a poor learning performance, an effect that was accentuated by co-administration of tramadol with nicotine, showing a poorer learning performance (Fig. 9a). In the probe trial, the time spent in the target quadrant was significantly lowered in tramadol and nicotine as well as tramadol/nicotine groups compared to control group (Fig. 9b) suggesting poor memory consolidation.

### 3.12. Influence of co-administration of nicotine with tramadol on aggressive behavior

Mice given tramadol showed no change in aggression scores



**Fig. 5.** Mice subjected to tramadol either alone or in combination with nicotine showed increment in cortical levels of NF-κB (a), TNF-α (b), nNOS (c) and iNOS (d). Data are expressed as mean of (6–8) experiments  $\pm$  SD. Statistical analyses were carried out by ANOVA followed by Tukey's Multiple Comparison Test. \*: significant from control, #: significant from tramadol, @: significant from nicotine at  $p < 0.05$ .

compared to their control counterparts (Fig. 9c), while those receiving nicotine displayed a prominent aggression score (7.3 fold) compared to control (Fig. 9c). However, mice given tramadol together with nicotine showed a decline in aggression score compared to nicotine group (Fig. 9c).

### 3.13. Impact of co-administration of nicotine with tramadol on social interaction test

Mice treated with tramadol showed no change in social interaction time compared to their control counterparts (Fig. 9d), on the other hand, nicotine decreased SI time by 53.8% compared to control group (Fig. 9d). Interestingly, mice treated with tramadol and nicotine showed a significant increase in SI time by 49.8% compared to nicotine group (Fig. 9d).

### 3.14. Effect of tramadol either alone or in combination with nicotine on assessment of anxiety-related behavior in OFT

#### 3.14.1. Thigmotaxis time and central square frequency

Mice receiving tramadol produced a significant increase in central square entry frequency (2.4 fold) paralleled by a significant decrease in thigmotaxis time (40.7%) compared to control, indicating anxiolytic effect of tramadol (Fig. 10a–b). On the contrary, animals that were given nicotine exhibited anxiogenic performance which was reflected

by no change in thigmotaxis time or central square entry frequency compared to control group (Fig. 10 a–b). Notably, mice receiving nicotine with tramadol showed a significant decrease in thigmotaxis time accompanied with a significant increase in central square frequency compared to nicotine group (Fig. 10 a–b).

#### 3.14.2. Ambulation and rearing frequencies

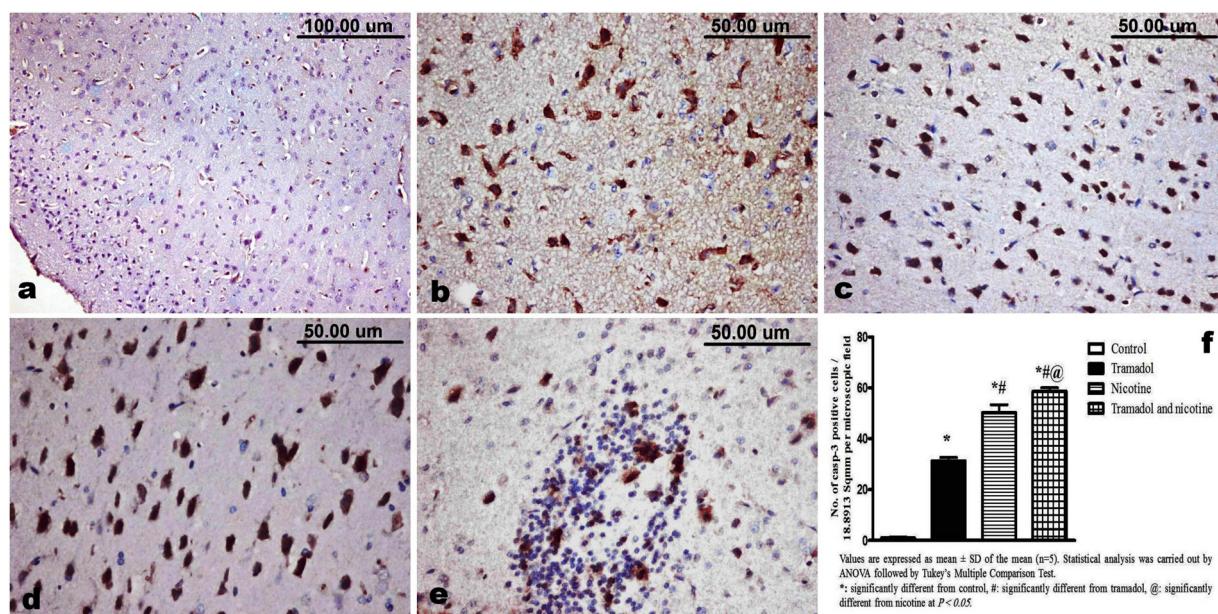
Injection of tramadol produced a significant increase in ambulation and rearing frequencies by 32 and 67%, respectively, compared to control (Fig. 10c–d). Mice given nicotine showed no significant change in ambulation and rearing frequencies compared to their control counterparts. Similarly, co-administration of tramadol with nicotine resulted in insignificant change in ambulation and rearing frequencies compared to control group (Fig. 10c–d).

#### 3.14.3. Grooming frequency

Both tramadol and nicotine alone or in combination produced significant increases in grooming frequency compared to control group (Fig. 10e).

### 3.15. Histopathological changes in cortices of mice subject to tramadol alone or in combination with nicotine

The brain sections of control mice showed normal histological structure (Fig. 11A/a). Generally it was noticed that nicotine



**Fig. 6.** Photomicrograph depicting caspase-3 immunohistochemical staining in cerebral cortex. Brown color (positive) indicates specific immunostaining for caspase-3. Control group showed negative expression of caspase-3 (a). Both tramadol and nicotine elevated expression of caspase-3 immunoreactive positive cells as shown by the intense brown staining particularly in nicotine (b and c). Furthermore, co-administration of nicotine with tramadol elevated expression of caspase-3 immunoreactive positive cells (d–e). The number of apoptotic cells (caspase-3 dependent) was determined by counting the number of positive cells in 5 randomly chosen microscopic fields at 40x objective magnification (f).

administration had more adverse effect than that caused by tramadol. Additionally, the combined administration had more deleterious effect than the sole administrations. The between-group comparisons were made in the same anatomic layers of the cerebral cortex (mostly layers II to V were the most affected with less severity in layers I and VI).

The brain sections of mice given tramadol (Fig. 11A/b) showed vacuolar degeneration and necrosis of some pyramidal neurons (Fig. 11A/c) with neuronophagia, the necrotic cells appeared darkly stained, structure-less without nuclei while others appeared faintly stained ghost-like. Apoptotic cells and few apoptotic bodies were noticed (Fig. 11A/d) with some dilated and congested blood capillaries. The brain of mice subjected to nicotine (Fig. 11A/e), showed congestion of the cerebral blood vessels, most of the pyramidal neurons appeared irregular darkly stained with pyknotic nuclei and surrounded by halos while others appeared shrunken necrotic and few appeared faintly stained ghost like with widely spread neuronophagia (Fig. 11A/f). The cerebral cortex of some animals showed hypercellularity (Fig. 11B/a), marked vacuolation of the neuropil with apparent neuronal vacuolar degeneration and necrosis as well as neuronophagia. Astrocytic reaction was noticed as swelling, edema and nuclear pyknosis. Apoptotic cells and bodies (Fig. 11B/b) were observed in all cases but of variable severity. Examination of brain tissue of the mice given tramadol and nicotine (Fig. 11B/c), revealed focal glial cells proliferation, marked shrinkage, vacuolar degeneration and necrosis of the neurons. Some

necrotic neurons appeared as remnants (Fig. 11B/d) while others appeared with pyknotic nuclei or appeared ghost-like without any nuclear structure accompanied with many apoptotic cells and bodies. Neuronophagia (Fig. 11B/e), hyperactivity of astrocytes and vacuolation of neuropil were all noticed. Few mice showed perivascular cuffing of the congested cerebral vessels (Fig. 11B/f).

#### 4. Discussion

In the current investigation we provide the first evidence, to authors' knowledge, regarding the divergent behavioral/neurotoxic effects of combining nicotine and tramadol. On one hand, tramadol in association with nicotine improved social interaction while decreasing anxiety and aggression linked to chronic administration of nicotine. However, on the other hand, this combination elicited negative effects regarding learning and memory consolidation. Tramadol and nicotine neurotoxic effects were investigated via assessment of different markers of oxidative stress, inflammation and apoptosis, in addition to histopathological examination of brain tissue.

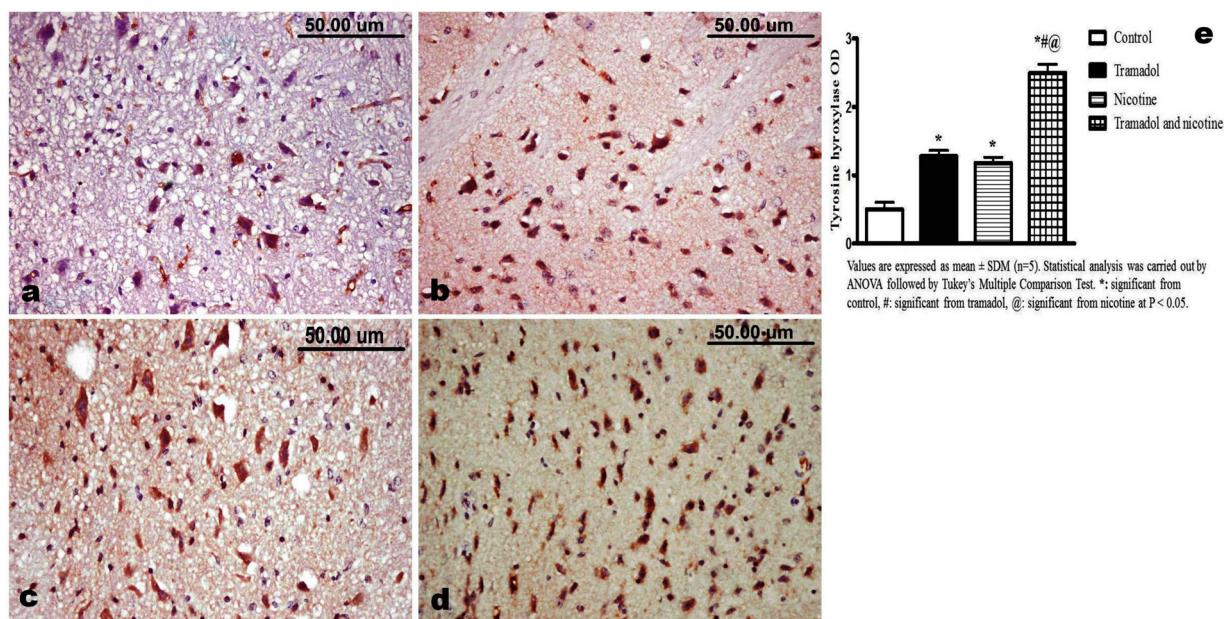
Prior to pursuing the neurochemical and neurobehavioral alterations induced by co-administration of tramadol and nicotine, it was imperative to determine whether nicotine had an effect on tramadol concentration in mice brain. Noteworthy, in the current investigation, nicotine induced a significant increase in tramadol concentration in

**Table 1**

Changes in cortical monoamines levels (DA, 5HT, NE, GLU, and GABA) induced by tramadol either alone or in combination with nicotine.

Groups	Parameters				
	DA (µg/g.tissue)	5 HT (µg/g.tissue)	NE (µg/g.tissue)	GLU (µg/g.tissue)	GABA (µg/g.tissue)
Control	0.95 ± 0.05	0.39 ± 0.05	0.48 ± 0.03	5.74 ± 0.3	6.33 ± 0.39
Tramadol	1.57 ± 0.03*	0.65 ± 0.06*	0.58 ± 0.04*	7.14 ± 0.42*	10.72 ± 0.59*
Nicotine	1.27 ± 0.08*#	0.34 ± 0.03#	0.69 ± 0.03*#	7.42 ± 0.30*	4.88 ± 0.50*#
TR. + NI.	1.53 ± 0.03*@	0.49 ± 0.05*#@	0.56 ± 0.05*#@	10.32 ± 0.64*#@	8.19 ± 0.19*#@

Data are expressed as mean of (6–8) experiments ± SD. Statistical analyses were carried out by ANOVA followed by Tukey's Multiple Comparison Test. \*: significant from control, #: significant from tramadol, @: significant from nicotine at  $p < 0.05$ .



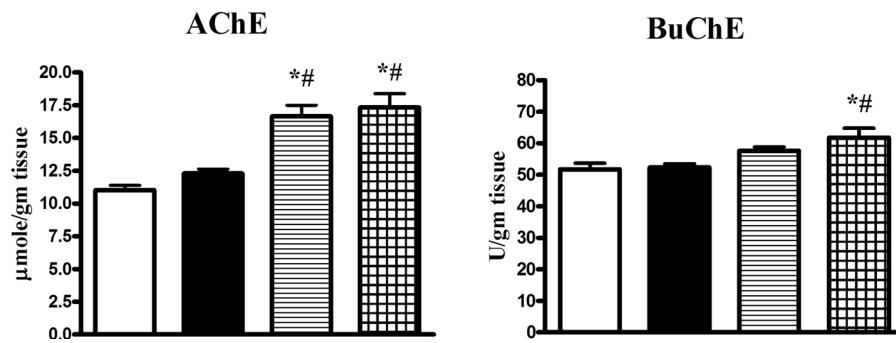
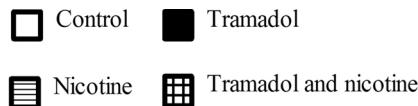
**Fig. 7.** Photomicrograph depicting TH immunohistochemical staining in mid brain. Brown color (positive) indicates specific immunostaining for tyrosine hydroxylase. Control group showed minimal immunostaining for TH (a). Both of tramadol and nicotine elevated expression of TH (b and c). Moreover, co-administration of tramadol with nicotine elevated expression of TH as shown by the intense brown staining (d). Quantitative image analysis for immunohistochemical staining expressed as optical densities (OD) across 10 different fields for each mouse section (e).

mice brain after 30 min of tramadol injection. The increment in tramadol concentration induced by its co-administration with nicotine might lend a plausible explanation for the heightened mortality seen with the combination. The current findings can be explained in the light of study of Wang et al. (2015) who reported that nicotine altered tramadol metabolism and increased  $C_{max}$  of tramadol and  $M_1$  in cerebrospinal fluid in rats; an event that would be attributed to induction of brain CYP<sub>450</sub> 2D enzyme by nicotine and enhance tramadol metabolism. Moreover, the present results are supported with the study of Solarino et al. (2010) who reported multidrug poisoning case involving tramadol and nicotine and attributed the fatality to that nicotine enhanced the effect of tramadol, suggesting that this combination may be toxic in large doses.

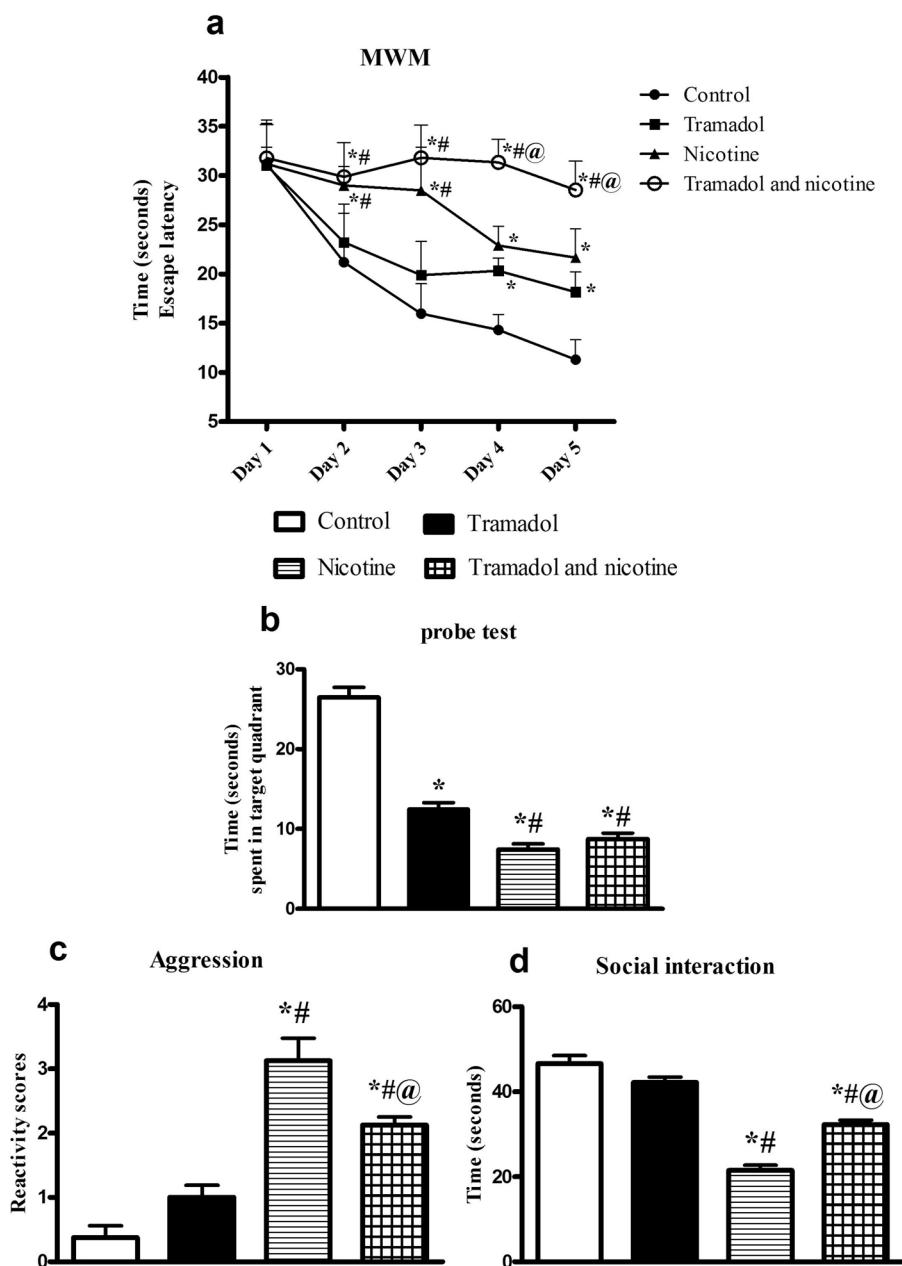
In the present study, a significant increase in cortical oxidative/nitrosative stress of mice subjected to tramadol and nicotine alone or in combination, reflected by increased TBARS and NO paralleled by decreased NPSHs. Noteworthy, microglia, the brain resident macrophages presents one source for the increased ROS (Carniglia et al., 2017).

Indeed, the present investigation outlines increased microglial immunoreactivity as manifest by enhanced CD68 positive cells in mice receiving either treatment alone or in combination. These results are consistent with several lines of evidence which indicated that repeated administration of tramadol (Abdel-Zaher et al., 2011) and nicotine (Bhagwat et al., 1998; Gumustekin et al., 2003; Hritcu et al., 2009) increased oxidative stress in the brain. Furthermore, DA auto-oxidation could afford a second plausible theory explaining oxidative stress accompanied with most of drugs of abuse (Cunha-Oliveira et al., 2013). DA quinones, which are electron-deficient molecules, can readily react with cysteinyl residues of proteins, such as GSH leading to glutathione depletion (Miyazaki and Asanuma, 2008); events that are supported by findings from this present report. Indeed, many studies reported that nicotine administration induced apoptosis in brain via increase of oxidative stress and moreover, nicotine was found to produce a remarkable loss of neurons in mid brain and cortex of adolescent rodents (Ferreira and Winterer, 2009; Hritcu et al., 2009).

The production of reactive nitrogen species (RNS) such as the



**Fig. 8.** Tramadol alone had no effect on cortical acetyl (a) and butyryl (b) cholinesterases while mice receiving nicotine alone showed increase in cortical acetylcholinesterase. Furthermore mice given nicotine with tramadol showed a significant increase in both enzymes. Data are expressed as mean of (6–8) experiments  $\pm$  SDM. Statistical analyses were carried out by ANOVA followed by Tukey's Multiple Comparison Test. \*: significant from control, #: significant from tramadol at  $p < 0.05$ .

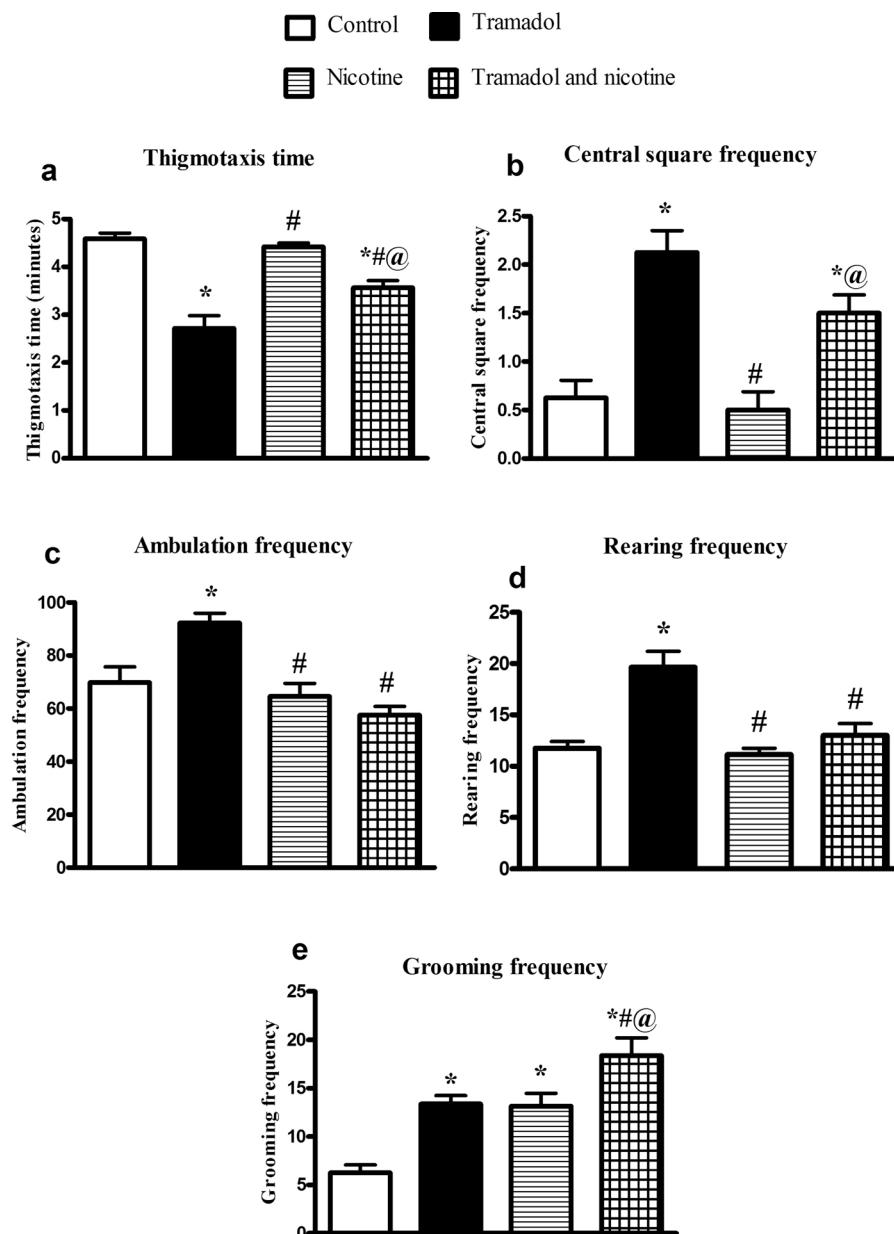


**Fig. 9.** Mice given tramadol and nicotine alone or in combination showed a disturbed performance in MWM (a); the time spent in the target quadrant was significantly less in groups receiving the aforementioned drugs in probe test (b); tramadol alone showed no change in defensive aggression and social interaction tests (c–d), nicotine treated mice displayed increased aggression score and decreased (SI) time (c–d), however mice given tramadol in addition to nicotine showed decrease in aggression and increase in (SI) time compared to nicotine per se (c–d). Data are expressed as mean of (6–8) experiments  $\pm$  SD. Statistical analyses were carried out by two way ANOVA followed by Bonferroni post test for MWM learning curve (a) and one way ANOVA followed by Tukey's Multiple Comparison Test for probe test (b), aggression (c), social interaction (d). \*: significant from control, #: significant from tramadol, @: significant from nicotine at  $p < 0.05$ .

radical nitric oxide (NO) and its metabolite peroxynitrite ( $\text{ONOO}^-$ ) present another form of cellular stress (Hastings, 2009). In fact, the current investigation demonstrates increased NO by nicotine and/or tramadol subsequent to enhanced nNOS/iNOS content. Worthy of note, microglial cells produce not only NO but also a variety of proinflammatory cytokines (Carniglia et al., 2017). Certainly, the present study showed a significant increase in iNOS and TNF- $\alpha$  levels in cortices of mice subjected to tramadol and nicotine alone or in combination. Worth mentioning, the increase in case of tramadol/nicotine combination was significant compared to tramadol or nicotine alone. The induction of these inflammatory mediators is mediated by several different pathways, one important pathway is through NF- $\kappa$ B (Karin, 2005). In fact, the present findings display augmented NF- $\kappa$ B, which is considered to be redox sensitive transcription factor (Gloire et al., 2006) that could have been activated by the present increase in ROS. NF- $\kappa$ B influences the expression of genes encoding proinflammatory cytokines in microglial cells like TNF- $\alpha$  and iNOS as well (Mattson and Camandola, 2001). In addition, TNF- $\alpha$  may indirectly increase iNOS in an NF- $\kappa$ B independent pathway thus adding to the proxidant milieu

(Nassar et al., 2015). Worth mentioning, the increment in TNF- $\alpha$  observed in this study could lend credence to the observed reduction in body weight seen in the current investigation with the three different regimens. Certainly, increased serum TNF- $\alpha$  inhibits the uptake of circulating free fatty acids and accelerates lipolysis in adipose tissue (Kirana et al., 2011; Georgy et al., 2013).

The current investigation displays an increased number of Casp-3 positive cells in groups receiving either tramadol or nicotine alone or in combination. Noteworthy, the observed increment in ROS as well as proinflammatory cytokines induce apoptotic cascades through intrinsic and extrinsic pathways (Dong et al., 2009). Moreover, the current investigation displays an increment in GLU, iNOS and nNOS. Indeed glutamatergic neurotransmission has been implicated in several processes involved in drug addiction, including reinforcement, sensitization, habit learning, craving, and relapse (Tzschentke and Schmidt, 2003). In a feed-forward cycle, GLU induced excitotoxicity is caused by a massive influx of extracellular  $\text{Ca}^{2+}$  resulting from the overactivation of the N-methyl- D-aspartate (NMDA) GLU receptors which further contributes to stress burden and hence Casp-3 activation (Zhang and



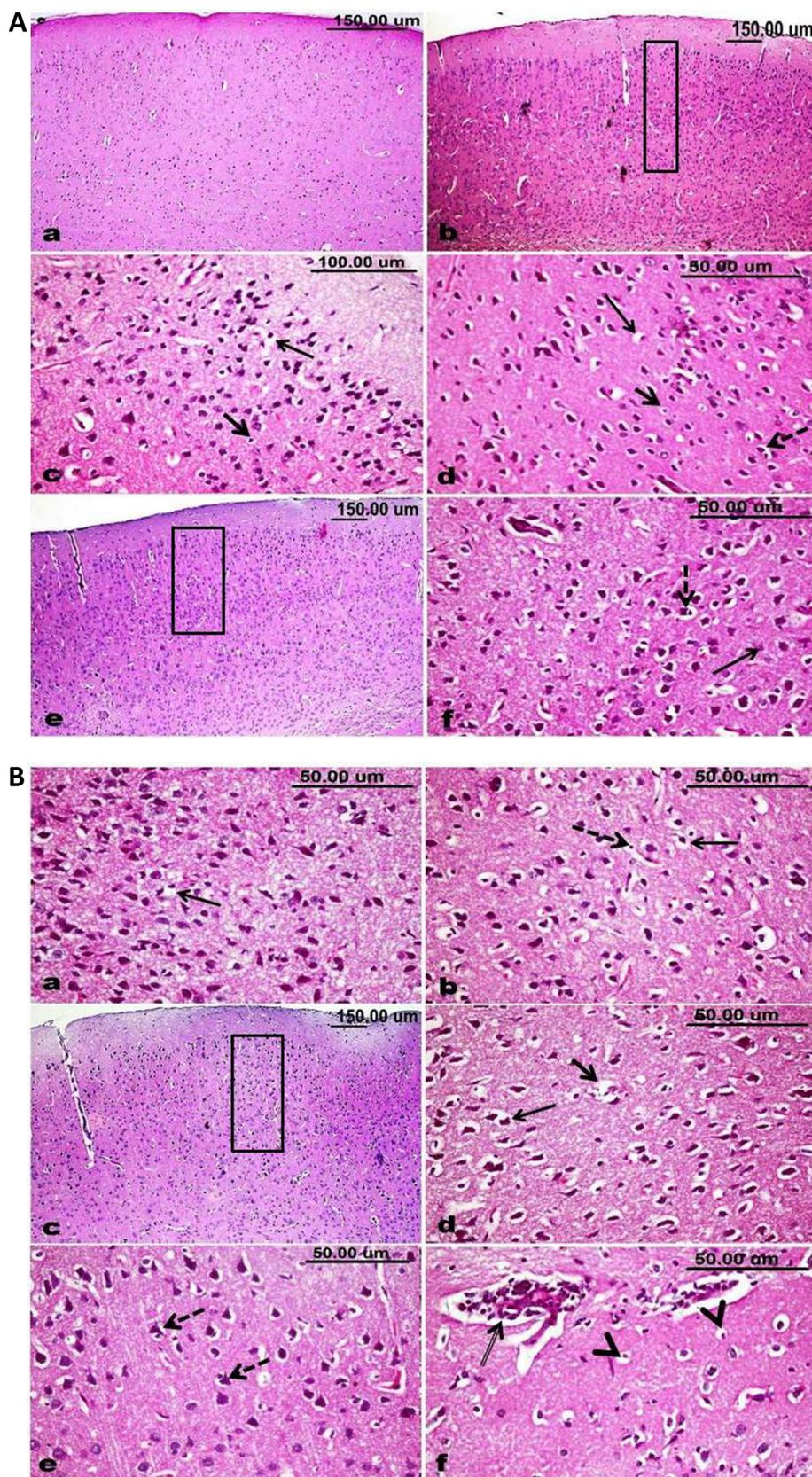
**Fig. 10.** Tramadol produced a significant decrease in thigmotaxis time (a) paralleled with increase in central square frequency (b) in addition to increase in ambulation (c), rearing (d) and grooming (e) frequencies. Nicotine alone induced a significant increase in grooming frequency (e) and had no change on other parameters (a-d). Mice receiving tramadol with nicotine displayed a significant decrease in thigmotaxis time (a) accompanied with increase in central square frequency (b) compared to nicotine alone, in addition to a significant increase in grooming frequency (e) compared to tramadol or nicotine. Data are expressed as mean of (6–8) experiments  $\pm$  SD. Statistical analyses were carried out by ANOVA followed by Tukey's Multiple Comparison Test. \*: significant from control, #: significant from tramadol, @: significant from nicotine at  $p < 0.05$ .

(Bhavnani, 2006). Consequent to the excitotoxicity exerted by GLU, the rise intracellular  $\text{Ca}^{2+}$  can stimulate nNOS through interaction with calmodulin resulting in massive increase in NO level, which in turn can react with  $\text{O}_2^-$  to form toxic ONOO<sup>-</sup> (Parathath et al., 2007; Dong et al., 2009; Cunha-Oliveira et al., 2013) to further exacerbate the free radical pool in addition to the observed changes seen with iNOS; thus implicating both isoforms in the damage exerted by tramadol and nicotine either alone or in combination.

The aforementioned abnormalities and biochemical data were further confirmed by histopathological examination of the brain cortices. The brain sections of mice receiving tramadol showed vacuolar degeneration and necrosis of some pyramidal neurons in addition to few apoptotic bodies. These histopathological changes are consistent with those reported by Atici et al. (2004) who found that chronic use of tramadol caused neuron degeneration which may lead to cerebral dysfunction. Moreover, consistent with oxidative/nitroactive stress, exaggerated inflammation and excitotoxicity reported herein following nicotine administration, the cerebral cortex of such mice showed hypercellularity, marked vacuolation of the neuropil, astrocytic reaction and apoptotic bodies. Furthermore, administration of tramadol with

nicotine caused focal glial cells proliferation, hyperactivity of astrocytes, vacuolar degeneration and necrosis of the neurons. These results support our findings concerning activation of microglial cells by ROS induced by tramadol and nicotine which sequentially lead to release of inflammatory cytokines/superoxide anion and at last apoptosis.

Dopaminergic neurons are prone to oxidative stress due to their high rate of oxygen metabolism, low levels of antioxidants, and high iron content (Halliwell, 1992). Paradoxically, this study showed that administration of tramadol and nicotine either alone or in combination resulted in a significant increase in cortical DA level, paralleled with the increased expression of TH in midbrain region. Our results are supported by study of Nakamura et al. (2008) which reported, that tramadol increased DA level via activation of  $\mu$ -opioid receptors, expressed in ventral tegmental area (VTA) in midbrain region and potentiation of mesolimbic pathway. Other studies also reported that nicotine can stimulate mesocorticolimbic system and enhance DA release by direct stimulation of nicotinic receptors in VTA (Koob and Le Moal, 2008; Cachope and Cheer, 2014). Furthermore, experimental evidence outlines a co-modulatory effect for that nicotine and opioids, where nicotine was found to induce DA release through activation of  $\mu$ -opioid receptors in



**Fig. 11. A:** Photomicrographs depicting the histological appearance of cerebral cortices (CX) of control mice showing normal histological structure (a). Mice given tramadol (20 mg/kg) (b–d) showing; neuronal vacuolar degeneration (arrow), few apoptotic bodies (short arrow) and necrosis with neuro-nophagia (dashed arrow) (c–d). Mice subjected to nicotine (0.25 mg/kg) (e–f) showing; darkly stained neurons with pyknotic nuclei, others shrunken necrotic and few ghost like (arrow) with marked neuronophagia (dashed arrow) (f) (H&E). **B:** CX of mice subjected to nicotine (0.25 mg/kg) (a–b) showing; hypercellularity (a), apparent neuronal vacuolar degeneration (arrow) and necrosis as well as apoptotic cells and bodies (dashed arrow) (b). CX of mice given combined tramadol and nicotine (c–f) showing; marked shrinkage and necrosis of the neurons (arrow) with remnants of necrotic cells (short arrow) (d) and marked neuronophagia (dashed arrow), neuronal vacuolar degeneration (e), many apoptotic cells and bodies (arrow head) and perivascular cuffing (double-line arrow) of the cerebral vessels (f) (H&E).

the VTA (Tanda and Di Chiara, 1998; Skurtveit et al., 2010). In addition, another study highlighted the existence for a mechanistic overlap between opiates and nicotine within DA reward pathway (Britt and McGehee, 2008). Consequently, our results may explain one of the

possible mechanisms underlying reward, addiction and dependence regarding tramadol and nicotine via potentiation of mesocortical pathway and increase DA levels.

However, one might argue against the discrepancy of enhanced

Casp-3 versus the preservation of DA neurons. Albeit, evidence suggests that an increment in GLU was observed consequent to Casp-3 that further contributes to increased addiction (Kenny, 2011) and hence increased DA content consequent to TH expression; events seen in all treatments in the present study.

Cholinergic neurons of the basal forebrain with its extensive cortical projections play a crucial role in cognitive and behavioral functions (Giacobini, 2003). In our study, mice subjected to nicotine showed a significant increase in AChE level, whereas mice receiving tramadol and nicotine showed a significant increase in both AChE and BuChE activities; corroborating with the disrupted performance observed in MWM test and memory consolidation. Indeed, a study by (Hu et al., 2009) revealed increased AChE in relation to increase Casp-3 expression and apoptosis in brain. Accordingly, in the present investigation the surge in AChE accompanied with the enhanced expression of Casp-3 in nicotine and nicotine/tramadol treated mice lend credence to the involvement of apoptosis in cognitive impairment induced by nicotine or tramadol/nicotine. On a closer look, AChE was not elevated with tramadol alone which would seemingly oppose the changes in MWM seen with the sole tramadol administration. One plausible explanation for this observation might be attributed to the present enhancement of cortical 5 HT and GABA, which have been shown previously to impair memory function (Fontana et al., 1995; Hosseini-Sharifabad et al., 2016).

The cortical area is not only involved in reward processing and cognitive functions, but also moderates social behavior, mood as well as anxiety (Martin et al., 2009). Noteworthy, such disorders are mediated by a plethora of neurotransmitter/amino acids disruptions. In the present study, mice subjected to tramadol showed a significant increase in cortical serotonin, NE and GABA levels. Moreover, tramadol treated mice showed anxiolytic behavior in OFT which was manifested by significant decrease in thigmotaxis time paralleled with increase in central square frequency. In addition, tramadol showed a significant increase in ambulation, rearing and grooming frequencies. The current findings are supported by study of Gholami et al. (2014) who reported that chronic tramadol re-exposure resulted in marked anxiolytic-like behaviors due to the ability of tramadol to inhibit 5 HT and NE re-uptake in brain (Faron-Gorecka et al., 2004). Worthy of note, the role of GABA has long been regarded as central to the regulation of anxiety and this neurotransmitter system is the target for many drugs used for treatment of anxiety disorders (Nuss, 2015).

Nicotine anxiogenic like effects could be mediated by enhancement of the release of some neurotransmitters such as, GLU and NE which are known to facilitate stress/anxiety related behavior (Balerio et al., 2005). Consistent with previous findings, in the current investigation, nicotine exposed mice showed a significant increase in cortical NE and GLU levels paralleled with decrease in GABA levels. Noteworthy, studies reported that decreased GABA levels are associated with mood and anxiety disorders (Kent et al., 2002). These changes in neurotransmitters and GLU levels were reflected by increased anxiogenic behavior in OFT as manifested with increase thigmotaxis time paralleled with decrease central square frequency. In addition, there was a significant increase in grooming frequency reflecting the increase in DA level (Kalueff et al., 2016).

HT and NE play a crucial role in modulating aggressive behavior. Elevated NE or decreased GABA levels may develop aggression (Eichelman, 1990). Moreover, increased excitatory glutaminergic activity is associated with social anxiety disorders (Martin et al., 2009). By the same token, mice receiving nicotine showed aggressive behavior and decrease in social interaction which may be attributed to decrease GABA levels and step up in NE and GLU levels. Interestingly, mice subjected to tramadol and nicotine showed a significant increase in cortical 5 HT and GABA levels at the same time as decreasing in NE level compared to nicotine per se and this was reflected in behavior tests as decreased in anxiety and aggressive behavior in addition to improvement in social interaction test compared to mice subjected to nicotine only. However, tramadol alone had no effect on defensive

aggression or social interaction tests.

Taken all together; findings from the present study provide compelling evidence for neurotoxic effects associated with co-administration of tramadol and nicotine despite enhanced reward manifested as increased DA and TH. Indeed, neurotoxicity is mediated through increase oxidative/nitrosative stress and enhanced levels of NO via induction of nNOS consequent to increments in GLU and iNOS downstream from NF- $\kappa$ B, TNF- $\alpha$  leading to neuroinflammation and at last apoptosis. Correlatively, the neuroinflammation and Casp-3 activation may contribute to the memory deficits induced by tramadol and nicotine. However, administration of tramadol and nicotine improved social interaction while decreasing anxiety and aggression associated with chronic administration of nicotine. Hence, many smokers tend to abuse tramadol as a phenomenal behavior, to reduce aggression and anxiety linked to nicotine while enhancing reward, however, such combination exacerbated neurotoxic effects and elicited negative effects regarding learning and memory.

## Conflict of interest

None.

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