Research Article

A study of the neurotoxic effects of tramadol and cannabis in adolescent male albino rats

Ola E. Nafea1*, Iman A. ElKhishin1, Othman A. Awad1, Dalia A. Mohamed2

1Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Zagazig University, Zagazig, Sharkia, Egypt
2Department of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Zagazig, Sharkia, Egypt

Received: 29 May 2016
Accepted: 21 June 2016

*Correspondence:
Dr. Ola E. Nafea
E-mail: olnafea@zu.edu.eg

Copyright: © the author(s), publisher and licensee Medip Academy. This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABSTRACT

Background: Adolescence is a critical period for cerebral development. Exposure to addictive substances during this phase leads to various alterations in brain functions that persist into adulthood. The present study was designed to study the neurotoxic effects of tramadol and cannabis, alone and in combination, in adolescent male albino rats by studying their behavioral, biochemical, and histopathological neurotoxic effects and their long–term consequences after withdrawal.

Methods: For this purpose, 132 adolescent male albino rats were divided into 5 groups (22 rats/ group). Group I (negative control), received only regular diet and tap water to measure the basic parameters, Group II (positive control; IIA&IIB): IIA, gavaged with normal saline. IIB, gavaged with olive oil. Group III (tramadol), gavaged with tramadol (42, 84 and 168 mg/kg/day) in the first, second and third ten days of the study respectively. Group IV (cannabis), gavaged with hashish extract (92, 184 and 368 mg/kg/day) in the first, second and third ten days of the study respectively. Group V (tramadol+cannabis), gavaged with tramadol and hashish extract in the same doses as Group III&IV. By the end of the first month, the half number of rats was subjected to performing behavior tests. Specimens from the brain were taken for performing biochemical and histopathological studies. All remaining rats were held for another 4 weeks non–dosing spontaneous recovery period after withdrawal of the treatment and were evaluated again by the same previous parameters.

Results: Abuse of tramadol or cannabis, alone and in combination, caused antidepressant effect (sucrose preference test), impaired spatial memory (Morris water maze), elevated serotonin levels in the cerebral cortex and hippocampus, induced oxidative stress (significantly elevated malondialdehyde level and reduced catalase activity) as well as deleteriously altered brain histopathology and marked increase in brain Caspase–3 expression. However, abuse of both tramadol and cannabis conferred more antidepressant effect but more neurotoxic effect. After withdrawal, the antidepressant effect was reversed, no improvement of the spatial memory, marked depletion of 5–HT, more improvement in antioxidants and apoptotic markers and incomplete regression of brain histopathological alteration resulted.

Conclusions: Abuse of tramadol and cannabis, alone and in combination, induced neurotoxicity which proved behaviorally, biochemically and histopathologically.

Keywords: Adolescence, Opioid, Cannabinoid, Neurotoxicity, Sucrose preference, Morris water maze

INTRODUCTION

“Drug addiction is a chronic relapsing brain disorder, characterized by the compulsion to seek and take drugs, a loss of inhibition in the ability to control amount of intake and the development of a negative hedonic state when access to drug is prohibited”1. Addiction also comprises craving for the substances and, in some cases,
involvement in risky behaviors that can lead to death.2 The brain is particularly sensitive to toxicity, because it is one of the most metabolically active tissues. The neurotoxic effects of drugs of abuse are commonly associated with oxidative stress, mitochondrial dysfunction, apoptosis and inhibition of neurogenesis, in addition to other mechanisms.3,4 Adolescence is a critical phase for cerebral development. Exposure to addictive substances during this phase leads to various alterations in brain functions that can be translated into functional consequences throughout life.5,7

Tramadol is a common prescription pain reliever that is structurally similar to morphine and codeine— with its analgesic effects identified as a mu–receptor agonist. Tramadol was originally considered to possess a much better safety profile than other opioid analgesics.8

Currently, tramadol abuse has become a primary concern all over the world during the last few decades.3,9 Tramadol abuse is associated with addictive behaviors among adolescents.10,11 Tramadol addiction is recognized to cause adverse and in some cases lethal health effects.12

Cannabis, which is derived from the plant Cannabis sativa, has been tangled with human culture from the dawn of history.13 Cannabis is broadly supposed as a safe recreational drug and its use is increasing among adolescents. However, repeated exposure to cannabis during adolescence may have unfavorable effects on brain resting functional connectivity, intelligence, and cognition.14 Cannabis is the most widely used illicit substance, with an estimated number of 119–224 million users worldwide.15,16 Cannabis has several effects on multiple organ systems including the CNS. Delta–9– tetrahydrocannabinol is the main psychoactive constituent of cannabis, and most, if not all, of the effects associated with the use of cannabis are caused by Δ–9–THC.17–19

The regular use of marijuana during adolescence is of specific concern, since use by this age group is associated with an increased possibility of deleterious consequences.20,21 Substance abuse in Egypt is an increasing public health concern. The Anti-Narcotic General Administration, stated that the narcotics problem costs the Egyptian economy around 800 million dollars annually.22

The present study was designed to study the neurotoxic effects of tramadol and cannabis, alone and in combination, in adolescent male albino rats by studying their behavioral, biochemical, and histopathological neurotoxic effects and their long–term consequences after withdrawal.

METHODS

Drugs and chemicals

Tramadol

Commercial available tramadol hydrochloride capsules were purchased from October Pharma S.A.E., Egypt, under license of German Grunenthal Company.

Cannabis

Hashish was obtained, after taking permission, from the Ministry of Justice.

Serotonin

Serotonin hydrochloride (standard for HPLC) was purchased from Sigma/Aldrich Chemical Company.

Preparation of Hashish extract

The crude material of hashish was analyzed for its common contents by gas chromatography (Agilent 5970 GC/MS) and was found to contain 5% of Δ–9–THC, 6.2% cannabidiol and 4.16% cannabidiolic acid.

The hashish extract was prepared by soaking hashish for 24 hours in ether. After removing the ether, the resin was weighed, dissolved in ether and then an amount of olive oil was added, so that the final product, after removing the ether, contained 0.5 g of resin in 5 ml of olive oil.23

Animals and treatments

One hundred and thirty two adolescent male albino rats weighing 60–100 g were included in the study, and were obtained from the Animal House of Faculty of Medicine, Zagazig University. Before commencing the experiment, all rats were subjected to 7 days period of passive preliminaries in order to adapt themselves to their new environment, to ascertain their physical well–being and to exclude any diseased rats. They were housed in a temperature–controlled and light–controlled room (12–h light/dark cycle), with free access to food and water. The adolescence in rodents starts from postnatal day (PND: 28+) while the adulthood starts from (PND: 60+).24–26

All animals received human care in compliance with the Animal Care Guidelines of the National Institutes of Health and the Ethical Committee for scientific research approved the design of the experiments. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The period of the study extended for 2 months (the first month was for the treatment and the second month was for the withdrawal).
**Experimental design**

The adolescent rats were divided into five groups as follows:

*Group I (negative control group)*: It consisted of 22 adolescent male albino rats. Each rat of that group was given regular diet and tap water to measure the basic parameters.

*Group II (positive control group)*: It consisted of 44 adolescent male albino rats. Rats in that group were subdivided equally into 2 subgroups:

1. *Subgroup IIA (normal saline group)*: Each rat of that group received 1ml of normal saline 0.9% NaCl (dissolvent for tramadol) once daily orally by gavage for 4 weeks.

2. *Subgroup IIB (olive oil group)*: Each rat of that group received 1 ml of olive oil (dissolvent for hashish) 23once daily orally by gavage for 4 weeks.

*Group III (tramadol group)*: It consisted of 22 adolescent male albino rats. Each rat of that group received 42, 84 and 168 mg/kg/day tramadol in the first, second and third ten days of the study respectively orally by gavage. The starting dose of tramadol (42 mg/kg/day) given to rats is equivalent to the human dosing regimen of 400 mg/day on body surface area conversion (animal dose (mg/kg) = human equivalent dose (mg/kg) × 6.2), assuming that an adult person weighs 60 kg. Rats received progressively increasing doses. 25-29

*Group IV (Cannabis group)*: It consisted of 22 adolescent male albino rats. Each rat of that group received 92, 184, 368 mg/kg/day of hashish extract in the first, second and third ten days of the study respectively orally by gavage. The starting dose of hashish extract (92 mg/kg/day) given to rats is equivalent to the human dosing regimen of 900 mg/day based on body surface area conversion (animal dose (mg/kg) = human equivalent dose (mg/kg) × 6.2), assuming that an adult person weighs 60 kg. 28,30 The previous doses of hashish represent moderate to heavy exposure to THC in humans. 31 Rats received progressively increasing doses. 29

*Group V (Tramadol + Cannabis group)*: It consisted of 22 adolescent male albino rats. Each rat of that group concurrently received tramadol and hashish extract in the same doses as group III&IV.

By the end of the first month of the experiment, the half number of rats from each group was subjected to:

1. Performing behavior tests (sucrose preference test and Morris water maze test).

2. Then rats were anaesthetized and sacrificed, and then specimens from the brain were taken for performing biochemical and histopathological study.

All remaining rats were held for another 4 weeks non–dosing spontaneous recovery period after withdrawal of the treatment and were evaluated again by the same previous parameters.

**Behavioural assessment**

*Sucrose preference test*

This test has been extensively validated by anti-depressant drugs. Sucrose is highly preferable to rats; hence, decreases in consumption of sucrose reflect a decreased sensitivity to normally rewarding stimuli, anhedonia, a major symptom of depression. 32

*Procedure*

The sucrose preference test was performed in the animal’s individual cages, where rats were given a 48–h (Postnatal date: PND 58 and 59& 88 and 89) two bottle exposure each cage was supplied with two identical graduated water bottles, each containing 250 ml of water. On the next day test, regular water in one of the bottles was replaced with 2% sucrose solution 33to avoid a potential locational drinking preference, the position of the bottles was changed after 24 hours. At the end of the test, the preference for the sucrose solution was calculated as the percentage of sucrose solution ingested relative to the total intake. 5

SUCROSE PREFERENCE (SP) was calculated according to the following equation:

\[
SP (\text{ml}) = \frac{\text{Sucrose intake (ml)}}{\text{Sucrose intake (ml) x water intake (ml)}} \times 100
\]

*Morris water maze test*

Morris water maze (MWM) test is one of the most common behavioral tasks used to determine hippocampal spatial memory deficits. 34

*Procedure*

The rats were trained in the standard Morris water maze (MWM) with a hidden platform. The rat must learn the location of a hidden platform by referring to visual cues placed around the room. The water maze consists of a circular pool (0.75 m diameter and 0.6 m height) filled to a depth of 25 cm with water. The escape platform is cube (10x10x10 cm) and submerged 2 cm below the surface of the water. A non–toxic white dye (starch solution) was added to the water to conceal the platform. Visual cues (images on white walls) were placed around the room and remained constant throughout testing. The Morris water maze task is a particularly sensitive task specific for hippocampal function. 35
The mobile phase formed of 20% methanol and 25 mM sodium acetate, adjusted to pH 5.1 with acetic acid. Fluorescence detection was made at 285 nm excitation and 340 nm emission wavelengths. Concentrations of serotonin were quantified by interpolating peak areas relative to those generated by a range of standards that were run in parallel to unknowns and calculated as µg of 5-HT to mg of wet tissue weight.  

Estimation of brain malondialdehyde (MDA) level and catalase (CAT) activity

Both brain MDA level and CAT activity were colorimetrically assayed according to the commercial available assay kits (Biodiagnostic for diagnostic reagents: Dokki, Giza, Egypt).

Histopathology study

Hematoxylin and eosiin staining

The brain was fixed in 10% formalin saline. After fixation, the brain was embedded in paraffin blocks and processed for the preparation of 5 µ. thickness sections. These sections were subjected to Hematoxylin and Eosin staining.

Immunohistochemical staining (IHS) for Caspase–3

Apoptosis was immunohistochemically localized using Caspase–3 antibodies. Paraffin sections (4 µm thick) were incubated with a rabbit monoclonal caspase–3 antibody (delivered from Lab Vision Laboratories– Cat. #: 1475–1) using the avidin biotin peroxidase method.

Morphometric study

Eleven non–overlapping fields for each specimen were selected randomly and analyzed. The optical density of Caspase 3 immunostaining was measured by using the NIH ImageJ (v1.50) program.

Optical density was calculated by the following formula:

\[
\text{Optical density} = \log(\text{max intensity/mean intensity})
\]

where max intensity = 255 for 8–bit images. The degree of immune reaction is indicated by optical density value; the darker it is, the larger the value is.

Statistical analysis

All the data (continuous data; normally distributed) were expressed as mean±SD. The data were analyzed by comparing means among groups using two–way ANOVA; the two factors were the treatment (tramadol, cannabis and tramadol+cannabis) and the duration (first month of treatment and second month of withdrawal). In the event of a significant ANOVA (P<0.05), subsequent Bonferroni post hoc tests or Bonferroni adjusted t–tests were used where appropriate.
RESULTS

Behavior assessment

Sucrose preference test

Two–way ANOVA of sucrose preference test (Figure 3A) showed significant overall effects of both treatment ($F_{3,80}=13.6; p<0.0001$) and duration ($F_{1,80}=562.89; p <0.0001$), as well as a significant interaction between these two main factors ($F_{3,80}=74.71; p <0.0001$).

Brain biochemical assessment

Serotonin (5–HT) levels in the cerebral cortex and hippocampus

Two–way ANOVA of 5–HT levels in the cerebral cortex and hippocampus (Table 1) showed significant overall effects of both treatment ($F_{3,80}=285.72; p<0.0001$) and duration ($F_{1,80}=1591.97; p <0.0001$), as well as a significant interaction between these two main factors ($F_{3,80}=297.11; p <0.0001$).

Brain malondialdehyde (MDA) level and catalase (CAT) activity

Regarding brain MDA levels, two–way ANOVA of brain MDA levels (Table 1) showed significant overall effects of both treatment ($F_{3,80}=174.95; p<0.0001$) and duration ($F_{1,80}=197.23; p <0.0001$), as well as a significant interaction between these two main factors ($F_{3,80}=23.63; p <0.001$).

Regarding brain CAT activity, two–way ANOVA of brain CAT activity (Table 1) showed significant overall effects of both treatment ($F_{3,80}=68.76; p<0.0001$) and duration ($F_{1,80}=159.87; p <0.0001$), as well as a significant interaction between these two main factors ($F_{3,80}=19.49; p <0.0001$).

Histopathology assessment

Hematoxycin and eosin staining

As regard negative control group; examination of H&E stained sections of rats’ brain showed normal cerebral cortex with its six layers (molecular, external granular, pyramidal, internal granular, ganglionic and the deepest multiform layer). Large pyramidal cells with basophilic cytoplasm, large vesicular nucleus and long apical dendrite were observed. Granular cells with large rounded vesicular nuclei were detected. The glial cells with small dense nuclei were also seen. Eosinophilic neutrophil forming the background for the cells was noted. The three layers of the hippocampus, molecular, pyramidal and polymorphic, were also shown (Figure 2).

As regard tramadol group; examination of H&E stained sections of rats’ brain showed neuronal cell disorganization and hypercellularity, as well as increased apoptotic cells and dilated blood vessels. Areas of severe haemorrhage and vacuolations of neutrophil were also seen. Red neurons, markers of apoptosis were evident. A red neuron was defined as a brightly stained neuron with scanty eosinophil cytoplasm, nuclear pyknosis, and perineural retraction spaces. Few nerve cells with
vesicular nuclei were observed. Glial cells with small nuclei were present (Figure 3).

As regard cannabis group; examination of H&E stained sections of rats’ brain showed congested meningeal blood vessels, irregular shrunken cells and vacuolated cytoplasm. The cells were surrounded by irregular wide spaces. Also, congested blood vessels dilated perivascular spaces were present (Figure 4).

Table 1: Effects of tramadol, cannabis and their combination on brain biochemical parameters (5–HT, MDA and CAT) after the first month of treatment and the second month of withdrawal.

| Parameter                        | Group               | First month of treatment (n=11) | Second month of withdrawal (n=11) |
|----------------------------------|---------------------|---------------------------------|----------------------------------|
| Cerebral cortex& hippocampus 5–HT (µg/mg. tissue) | Negative control   | 0.87±0.061                      | 0.90±0.061                      |
|                                  | TRM                 | 20.99±3.48<sup>a</sup>          | 0.48±0.11<sup>*</sup>           |
|                                  | CANN                | 18.66±3.37<sup>a</sup>          | 0.42±0.13<sup>*</sup>           |
|                                  | TRM+CANN            | 43.54±4.76<sup>abc</sup>        | 0.45±0.15<sup>*</sup>           |
| Brain MDA (nmol/g. tissue)       | Negative control   | 0.47±0.05                       | 0.51±0.05                       |
|                                  | TRM                 | 18.38±3.64<sup>a</sup>          | 9.14±2.30<sup>*</sup>           |
|                                  | CANN                | 15.48±2.30<sup>ab</sup>         | 7.16±1.36<sup>*</sup>           |
|                                  | TRM+CANN            | 21.36±3.70<sup>abc</sup>        | 10.04±1.21<sup>**</sup>         |
| Brain CAT (U/g)                  | Negative control   | 43.21±4.68                      | 43.48±4.67                      |
|                                  | TRM                 | 19.65±3.97<sup>a</sup>          | 35.04±4.74<sup>**</sup>         |
|                                  | CANN                | 21.55±4.45<sup>a</sup>          | 37.05±4.02<sup>**</sup>         |
|                                  | TRM+CANN            | 15.51±2.93<sup>abc</sup>        | 34.85±6.29<sup>**</sup>         |

All data were expressed as mean±SD. Two way ANOVA test with Bonferroni post hoc test (significant if p ≤0.05), <sup>a</sup> significant vs negative control group, <sup>b</sup> significant vs TRM, <sup>c</sup> significant vs CANN of the same period (treatment or withdrawal). <sup>*</sup> Significant vs the same group after the treatment period compared to the withdrawal period (Bonferroni adjusted t–test). TRM; Tramadol, CANN; Cannabis.

Figure 2: Shows a section of brain of negative control group. Panel (A) shows a delicate layer of pia matter (arrowhead) and the typical layered appearance of the cerebral cortex labelled I–VI as follows: I–Molecular layer; II–External granular layer; III–Pyramidal cell layer; IV–Internal granular layer; V–Ganglionic layer and VI–Multiform layer (H&E × 100). Panel (B) shows normal histodevelopment. Large pyramidal cells (arrows) with basophilic cytoplasm, large vesicular nucleus and long apical dendrite are observed. Granular cells (arrowhead) with large rounded vesicular nuclei are detected. The glial cells (curved arrows) with small dense nuclei are also seen. Note, eosinophilic neutrophil (asterisks) forming the background for the cells. The three layers of the hippocampus, Molecular (M), Pyramidal (P) and Polymorphic (PP), are also shown (H&E ×400). Panel (C) shows the normal negative reaction of brain tissue to the Caspase–3 antibody (Caspase immunostaining ×400).

Figure 3: Shows a section of brain of tramadol–treated group. Panel (A) shows neuronal cell disorganization and hypercellularity, as well as increased apoptotic cells (arrows) and dilated blood vessel (DV). Note areas of severe haemorrhage (HG). Vacuolations of neuropil (V) and few red neurons (arrowhead) are evident. (H&E x100). Panel (B) shows marked neuronal degeneration and extensive vacuolations (V). Red neurons (curved arrows) are evident. Few nerve cells have vesicular nuclei (arrowheads). Note glial cells with small nuclei. Increased apoptotic cells in area of the hippocampus (arrows) are observed (H&E x400). Panel (C) shows many neurons with strong positive reactions to the Caspase–3 antibody in their cytoplasm (Caspase immunostaining ×400).
Figure 4: Shows a section of brain of cannabis–treated group. Panel (A) shows congested blood vessels (BV) in meninges. Irregular shrunken cells and vacuolated cytoplasm (arrows) are seen. The cells are surrounded by irregular wide spaces (curved arrows). (H&E × 100). Panel (B) shows congested blood vessels (BV) and dilated perivascular space (arrow). Also, vacuolated cytoplasm (V) is seen (H&E × 400). Panel (C) shows strong positive reactions to the Caspase–3 antibody in the cytoplasm of most neurons (Caspase immunostaining ×400).

As regard tramadol+cannabis group; examination of H&E stained sections of rats' brain showed areas of severe hemorrhage in the hippocampus area. Neuronal loss with degenerative changes and gliosis were evident. Most nuclei were apoptotic and destructed were also seen (Figure 5).

Figure 5: Shows a section of brain of tramadol+cannabis–treated group. Panel (A) shows areas of severe haemorrhage (HG) in the hippocampus area (H&E × 100). Panel (B) shows neuronal loss with degenerative changes (N) and gliosis (G). Most nuclei are apoptotic and destructed (arrowheads) (H&E × 400). Panel (C) shows increased number of strongly positive reacted neurons (arrow). (Caspase immunostaining ×400)

As regard tramadol–withdrawn group; examination of H&E stained sections of rats' brain showed thick meninges. Some nuclei were apoptotic while other nuclei appear nearly normal were seen. Vacuolations and decreased thickness of pyramidal layer were evident. Some glial cells with large nuclei were noted. Nearly normal appearance of the hippocampus with its three layers, molecular, pyramidal ark nuclei and wide pale area of the cytoplasm were observed. Apoptotic neurons with pyknotic nuclei could also be seen (Figure 6).

Figure 6: Shows a section of brain of tramadol–withdrawn group. Panel (A) shows thick meninges (arrow). Some nuclei are apoptotic (arrowheads) while other nuclei appear nearly normal (H&E × 100). Panel (B) shows decreased thickness of pyramidal layer (*). Vacuolations (V) in the pyramidal layer are seen. Some glial cells appear with large dark nuclei and wide pale area of the cytoplasm (arrow). Apoptotic neurons with pyknotic nuclei can also be seen (curved arrows), (H&E × 400). Panel (C) shows few numbers of positively reacted neurons (Caspase immunostaining ×400).

As regard cannabis–withdrawn group; examination of H&E stained sections of rats' brain showed inflammatory cellular infiltration and vacuolated cytoplasm. An area of gliosis was seen. Thick men and polymorphic was observed (Figure 7).

Figure 7: Shows a section of brain of cannabis–withdrawn group. Panel (A) shows inflammatory cellular infiltration (IC). Vacuolations (V) are still evident. An area of gliosis (G) is seen. Thick meninges (arrow) are noted (H&E × 100). Panel (B) shows nearly normal appearance of the hippocampus with its three layers: Molecular (M), Pyramidal (P) and Polymorphic (PP) (H&E × 400). Panel (C) shows mild brown immunoreaction in the cytoplasm of few neurons (arrow) (Caspase immunostaining ×400).

As regard tramadol+cannabis–withdrawn group; examination of H&E stained sections of rats' brain showed inflammatory cellular infiltrations and thick congested oedematous meninges. Some red neurons were still seen. Areas of vacuolations were present (Figure 8).

**Immunohistochemical staining (IHS) & morphometric study**

Immunohistochemical examination of sections of the brain of all treated groups rats showed many neurons
with strong positive reactions to the Caspase–3 antibody in their cytoplasm.

Figure 8: Shows a section of brain of tramadol+cannabis--withdrawn group. Panel (A) shows inflammatory cellular infiltrations (IC) (H&E × 100). Panel (B) shows thick congested oedematous meninges (arrows). Some red neurons (arrowheads) are seen. Areas of vacuolations (V) are still evident (H&E × 400). Panel (C) shows strong positive brown reaction within cytoplasm of some neurons (arrow). Mild brown reaction is seen in the cytoplasm of most of neurons (arrowhead) (Caspase immunostaining ×100).

The immunohistochemistry results were correlated with the optical density of Caspase–3 positive cells while IHS examination of sections of the brain of tramadol and cannabis withdrawn rats showed few neurons with mild positive reactions to the Caspase–3 antibody in their cytoplasm. Tramadol+cannabis--withdrawan group showed strong positive brown reaction within cytoplasm of some neurons and mild brown reaction were evident.

Two–way ANOVA of the optical density of Caspase–3 immunoreactions in the brain tissue (Figure 3C) showed significant overall effects of both treatment (F3,80 =91.85; p<0.0001) and duration (F1,80 =44.30; p<0.0001), as well as a significant interaction between these two main factors (F3,80 =22.09; p<0.0001).

**DISCUSSION**

Adolescence stage is a crucial period in neurodevelopment. Adolescent substance users show abnormalities in neurocognition, brain structure and brain function.43

The findings of the present study showed that abuse of tramadol or cannabis alone caused antidepressant–like behaviour, impaired spatial memory, elevated 5–HT levels in the cerebral cortex and hippocampus, induced oxidative stress and apoptosis in brain tissue and deleteriously altered brain structure. Tramadol abuse had a potent antidepressant effect and a more potential to elicit oxidant stress than cannabis. However, abuse of both tramadol and cannabis conferred more antidepressant effect but more neurotoxic effect. Whereas following withdrawal period, the antidepressant –like behaviour was reversed, no improvement of the spatial memory was achieved, marked depletion of 5–HT resulted, more improvement in antioxidants and apoptotic markers was evident, as well as incomplete recovery of brain histopathological alteration.

The development of depression in animals is associated with the reduction in intake of a sucrose solution.44

The cerebral cortex is the brain region where serotonergic transmission has been involved in the development of depression and related disorders.55 In fact, serotonin is the most widely studied neurotransmitter in depression.46

Tramadol antidepressant effect is probably due to increase brain serotonin levels by two mechanisms; tramadol acts as a serotonin releaser and as a serotonin reuptake inhibitor.47,48

Additionally, Abdel–Salam et al proved that the repeated administration of cannabis extract increased brain levels of serotonin.49

The enhanced antidepressant effect observed in the present study after prolonged administration of tramadol with cannabis is probably a result of an additive interaction between them.50

Depression is a well–documented withdrawal symptom of tramadol and marijuana abstinence.51,52

The role of oxidative stress in the development of cognitive and memory impairment was proved by several research studies.53–55

Therefore, oxidative stress might be the potential mechanism of memory impairment of tramadol and cannabis. The present work demonstrated that abuse of tramadol, cannabis and their combination caused significant elevation in MDA (marker of lipid peroxidation) with reduction in the antioxidant (CAT) activity. However, a combination of them caused more worsening in the redox state than each one did separately.

In corroboration, Hosseini–Sharifabad proved that tramadol impaired memory when administered acutely or chronically.56 Similarly, Lubman et al stated that cannabis abuse had been commonly associated with increased rates of mental illness and cognitive dysfunction, predominantly among adolescent users.57

But in the current study, even after one month of abstinence, the improved oxidative stress wasn't associated with memory improvement.

Intact serotonergic neurotransmission alone can't lead to intact memory. This assumption is supported by previous research studies.58,59 They specifically investigated the destruction of serotonergic afferents to the hippocampus, and found no significant effects on spatial memory in the
water maze and T-maze. Interactions between cholinergic and serotonergic transmission in the hippocampus have been extensively studied. This fact could elucidate the increased serotonin levels in the cerebral cortex and hippocampus is not associated with improvement of memory during the first month of abuse in the present work. However, Normile et al showed that serotonin depletion enhanced learning.

Surprisingly, Sarikaya and Gülçin proved that serotonin possess antioxidant and radical scavenging capacity by different in vitro methodologies.

Subsequently, in the current study, increased serotonergic activity could be considered as a compensatory mechanism to scavenge free radicals generated by tramadol, cannabis and their concurrent abuse.

Finally, we examined the effect of tramadol and cannabis on the brain structure and on the apoptotic marker (Caspase–3) in the brain tissue. Tramadol or cannabis alone negatively altered brain histopathology. However, a combined abuse of both of them was associated with more pronounced altered brain histopathology and marked increase in Caspase–3 expression.

Caspase–3 activity is early detected in apoptosis, continues to increase as cells undergo apoptosis, and rapidly declines in late stages of apoptosis.

Tramadol–induced brain histopathological alterations were similar to the results of a study conducted by Ghoneim who reported that tramadol caused cortical layers disorganization, hypercellularity and increased apoptotic cells. As well, extensive cellular vacuolization, associated with dilated blood vessels and red neurons.

The partial recovery of brain tissue after tramadol withdrawal could be supported by Maschke who stated that gradual improvement of heroin–induced toxic leukoencephalopathy was observed after 4 weeks of heroin exposure and nearly complete recovery was noticed after 6 months of exposure.

Iversen stated that the brain is the organ mostly affected by administration of (THC) because its main effects are mediated through the cannabinoid receptors CB1 that densely concentrated e brain regions. Similarly, Yassa studied the effect of subchronic toxicity of cannabis leaves (bango) on brain of experimental rats and they found noticeable brain tissue affection in the form of irregular shrunken cells with dense nuclei and vacuolated cytoplasm in all layers.

Accordingly, in the current study, tramadol, cannabis and their simultaneous abuse induced–brain oxidative stress is a Caspase triggering based on biochemical data. The partial recovery of brain tissue after cannabis withdrawal could be explained by the function of endocannabinoid system (eCBs). In fact, release of endocannabinoids are ‘on demand’ in response to various events or stimuli. However, non–eCBs independent mechanism is superior in counteracting of neuropathological events.

The marked histopathological brain damage of adolescent rats received concurrent tramadol and cannabis could be explained by a super–additive or synergistic effects of both agents.

As well as, their abuse during adolescence induced long–term structural brain alteration in adulthood. Unfortunately, after withdrawal, the complete reversibility wasn't achieved. Simultaneous tramadol and cannabis abuse had the most dominant neurotoxic effects on the all studied parameters.

CONCLUSION

The overall results of the present study suggested that abuse of tramadol and cannabis, alone and in combination, induced neurotoxicity which proved behaviorally, biochemically and histopathologically. Abuse of tramadol and cannabis, alone and in combination, during adolescence caused behavior alteration in the form of antidepressant–like effect and impaired spatial memory. However, long–term behavioral consequences (depression and lasting memory impairment) continued in adulthood even after withdrawal. The significant elevation of MDA and drop in CAT activity, as well as augmented expression of Caspase–3 in brain tissue were indicators that abuse of tramadol, cannabis and combination of them had the potential to induce oxidative stress.

As well as, their abuse during adolescence induced long–term structural brain alteration in adulthood. Unfortunately, after withdrawal, the complete reversibility wasn't achieved. Simultaneous tramadol and cannabis abuse had the most dominant neurotoxic effects on the all studied parameters.

**Funding:** No funding sources  
**Conflict of interest:** None declared  
**Ethical approval:** The study was approved by the institutional ethics committee of Zagazig Faculty of Medicine, Egypt

**REFERENCES**

1. Gipson DC, Kalivas WP. Neural Basis of Drug Addiction. In: De Micheli D, Andrade MAL, da Silva AE, de Souza Formigoni OML, eds. Drug Abuse in Adolescence: Neurobiological, Cognitive, and Psychological Issues. Switzerland: Cham: Springer International Publishing; 2016:37–56.
2. Cadet JL, Bisagno V, Milroy CM. Neuropathology of substance use disorders. Acta Neuropathol. 2014;127(1):91–107.
3. Cunha–Oliveira T, Rego AC, Oliveira CR. Cellular and molecular mechanisms involved in the
neurotoxicity of opioid and psychostimulant drugs. Brain Res Rev. 2008;58(1):192–208.

4. Cunha–Oliveira T, Rego AC, Garrido J, Borges F, Macedo T, Oliveira CR. Neurotoxicity of heroin–cocaine combinations in rat cortical neurons. Toxicol. 2010;276(1):11–7.

5. Rubino T, Viganò D, Realini N, Guidali C, Braida D, Capurro V, et al. Chronic delta9-tetrahydrocannabinol during adolescence provokes sex–dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. Neuphysropsychopharmacol. 2008;33(11):2760–71.

6. Omotoso GO, Adeekeye MO, Ariyo AA, Ibiolu JO, Oyeyemi OA, Enaibe BU. Neurohistochemical studies of adolescent rats' prefrontal cortex exposed to prenatal nicotine. Ibnosina J Med Biomed Sci. 2014;6(1):25–30.

7. Jacobus J, Tapert SF. Neurotoxic effects of alcohol in adolescence. Ann Rev Clin Psychol. 2013;9:703–721.

8. Batta A. TRAMADOL–A drug to be used cautiously. Int J Curr Res Med Sci. 2016;2(2):11–7.

9. Abdel–Hamid IA, Andersson K–E, Waldinger MD, Anis TH. Tramadol abuse and sexual function. Sex Med Rev. 2016;4(3):235–46.

10. Taghaddosinejad F, Mehrpour O, Afshari R, Seghatoleslami A, Abdollahi M, Dart RC. Factors related to seizure in tramadol poisoning and its blood concentration. J Med Toxicol. 2011;7(3):183–8.

11. Karrari P, Mehrpour O, Balali-Mood M. Iranian crystal: a misunderstanding of the crystal–meth. J Res Med Sci. 2012;17(2),203–4.

12. Kusari S, Tatsimo SJ, Zühlke S, Spiteller M. Synthetic origin of tramadol in the environment. Angew Chem Int Ed Engl. 2016;55(1):240–3.

13. Bourne PG. Marijuana: Medical Applications, Recreational Use and Substance Abuse Disorders. In: Friedman, H.S. ed. Encyclopedia of Mental Recreational Use and Substance Abuse Disorders. Bourne PG. Marijuana: Medical Applications, Recreational Use and Substance Abuse Disorders. In: Friedman, H.S. ed. Encyclopedia of Mental Recreational Use and Substance Abuse Disorders. 2013;2(4):65–9.

14. Reagan–Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2008;22(3):659–61.

15. Atici S, Cinel L, Cinel I, Doruk N, Aktekin M, Akca A, et al. Opioid neurotoxicity: comparison of morphine and tramadol in an experimental rat model. Int J Neurosci. 2004;114(8):1001–11.

16. Osman AM, Shaalan M, Wagih I, Affifi M. Anticonvulsant effects of hashish in human epileptics. Egypt J Psychiatry. 1984;7:85–97.

17. Rubio P, De Fonseca F, Martín–Calderón JL, Del Arco I, Bartolomé S, Villanúa MaA, et al. Maternal exposure to low doses of Δ9-tetrahydrocannabinol facilitates morphine–induced place conditioning in adult male offspring. Pharmacol Biochem Behav. 1998;61(3):229–38.

18. Hong S, Flashner B, Chiu M, ver Hoeve E, Luz S, Bhatnagar S. Social isolation in adolescence alters behavioral changes in the forced swim and sucrose preference tests in female but not in male rats. Physiol Behav. 2012;105(2):269–75.

19. Montegia LM, Luikart B, Barrot M, Theobold D, Malkovska I, Nef S, et al. Brain–derived factor 1 and δ-opioid receptors promote spine formation in the adult spinal cord. Proc Natl Acad Sci USA. 2006;103(29):10945–50.

20. Scherma M, Dessi C, Muntoni AL, Lecca S, Satta V, Luchichia A, et al. Adolescent Δ9-tetrahydrocannabinol Exposure Alters WIN55, 212–2 Self-Administration in Adult Rats. Neuropsychopharmacol. 2016;41(5):1416–26.

21. Marie D, Fergusson DM, Boden JM. Links between ethnic identification, cannabis use and dependence, and life outcomes in a New Zealand birth cohort. Aust N Z J Psychiatry. 2008;42(9):780–8.

22. Yassa HA, Abd El Wahab AD, shehata MM, Abdel–Hady RH, Abdel–Aal KM. Subchronic toxicity of cannabis leaves on male albino rats. Hum Experimental Toxicol. 2009;29(1):37–47.

23. Miras C, Kephals T, Papadakis D. The effect of hashish extract on the norepinephrine in rabbit brain. Bull Narcoat. 1971;23:33–4.

24. Laviola G, Macrì S, Morley–Fletcher S, Adriani W. Risk–taking behavior in adolescent mice: psychobiological determinants and early epigenetic influence. Neurosci Biobehav Rev. 2003;27(1):19–31.

25. Quinn HR, Matsumoto I, Callaghan PD, Long LE, Arnold JC, Gunasekaran N, et al. Adolescent rats find repeated Δ9–THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. Neuropsychopharmacol. 2008;33(5):1113–26.

26. Marco EM, Adriani W, Ruocco LA, Canese R, Sadile AG, Laviola G. Neurobehavioral adaptations to methylphenidate: The issue of early adolescent exposure. Neurosci Biobehav Rev. 2011;35(8):1722–39.

27. Borgerding MP, Absher RK, So T–Y. Tramadol use in pediatric sickle cell disease patients with vasoo–occlusive crisis. World J Clin Pediatr. 2013;2(4):65–9.

28. Atici S, Cinel L, Cinel I, Doruk N, Aktekin M, Akca A, et al. Opioid neurotoxicity: comparison of morphine and tramadol in an experimental rat model. Int J Neurosci. 2004;114(8):1001–11.
neurotrophic factor conditional knockouts show gender differences in depression–related behaviour. Biol Psychiatry. 2007;61(2):187–97.
34. Morris R. Developments of a water–maze procedure for studying spatial learning in the rat. J Neurosci Methods. 1984;11(1):47–60.
35. West MJ. Regionally specific loss of neurons in the aging human hippocampus. Neurobiol Aging. 1993;14(4):287–93.
36. Hadad–Ophir O, Albrecht A, Stork O, Richter–Levin G. Amygdala activation and GABAergic gene expression in hippocampal sub–regions at the interplay of stress and spatial learning. Front Behav Neurosci. 2014;8:3.
37. Baraka A, ElGhony S. Study of the effect of inhibiting galanin in Alzheimer's disease induced in rats. Eur J Pharmaco. 2010;641(2):123–7.
38. Spijker S. Dissection of rodent brain regions. Neuroproteomics. 2011;57:13–26.
39. Cox BM, Shah MM, Cichon T, Tancer ME, Galloway MP, Thomas DM, et al. Behavioral and neurochemical effects of repeated MDMA administration during late adolescence in the rat. Prog Neuro–Psychopharmac Biol Psychiatry. 2014;48:229–35.
40. Krajewska M, Wang H–G, Krajewski S, Zapata JM, Shabaik A, Gascoyne R, et al. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase–3), a cell death protease. Cancer Res. 1997;57(8):1605–13.
41. Mustafa HN, El Awdan SA, Hegazy GA, Jaleel GAA. Prophylactic role of coenzyme Q10 and Cynara scolymus L on doxorubicin–induced toxicity in rats: Biochemical and immunohistochemical study. Indian J Pharmaco. 2015;47(6):649.
42. Squeglia LM, Jacobus J, Tapert SF. The influence of substance use on adolescent brain development. Clin EEG Neurosci. 2009;40(1):31–8.
43. Ozerov A, Bagmetova V, Chernysheva YV, Tyurenkov I. Comparison of the efficiency of adrenergic and antidepressants of various groups on the model of reserpine–induced depression in rats. Bull Exp Biol Med. 2016;160(5):649–52.
44. Arango V, Underwood MD, Mann JJ. Serotonin brain circuits involved in major depression and suicide. Prog Brain Res. 2002;136:443–53.
45. Darwish IE, Maklad HM, Diab IH. Behavioral and neuronal biochemical possible effects in experimental induced chronic mild stress in male albino rats under the effect of oral barley administration in comparison to venlafaxine. Int J Physiol Pathophysiol Pharmacol. 2013;5(2):128.
46. Grond S, Sablotzki A. Clinical pharmacology of tramadol. Clin Pharmacokinet. 2004;43(13):879–923.
47. Vazzana M, Andreani T, Fanguiero J, Faggio C, Silva C, Santini A, et al. Tramadol hydrochloride: pharmacokinetics, pharmacodynamics, adverse side effects, co–administration of drugs and new drug delivery systems. Biomed Pharmacother. 2015;70:234–8.
48. Abdel–Salam OM, Salem NA, El–Shamarka ME–S, Ahmed NA–S, Hussein JS, El–Khyat ZA. Cannabis–induced impairment of learning and memory: Effect of different nootropic drugs. EXCLI J. 2013;12:193.
49. Szkutnik–Fiedler D, Kus K, Balcerkiewicz M, Grzeskowiak E, Nowakowska E, Burda K, et al. Concomitant use of tramadol and venlafaxine–evaluation of antidepressant–like activity and other behavioral effects in rats. Pharmacol Rep. 2012;64(6):1350–8.
50. Senay EC, Adams EH, Geller A, Inciardi JA, Munoz A, Schnoll SH, et al. Physical dependence on Ultram®(tramadol hydrochloride): both opioid–like and atypical withdrawal symptoms occur. Drug Alcohol Depend. 2003;69(3):233–41.
51. Haney M. The marijuana withdrawal syndrome: diagnosis and treatment. Curr Psychiatry Rep. 2005;7(5):360–6.
52. Polydoro M, Schroder N, Lima MN, Caldana F, Laranja DC, Bromberg E, et al. Haloperidol– and clozapine–induced oxidative stress in the rat brain. Pharmacol Biochem Behav. 2004;78(4):751–6.
53. Schroder N, de Lima MN, Quevedo J, Dal Pizzol F, Roesler R. Impairing effects of chronic haloperidol and clozapine treatment on recognition memory: possible relation to oxidative stress. Schizophrenia Res. 2005;73(3):377–8.
54. Clausen A, Doctrow S, Baudry M. Prevention of cognitive deficits and brain oxidative stress with superoxide dismutase/catalase mimetics in aged mice. Neurobiol Aging. 2010;31(3):425–33.
55. Hosseini–Sharifabad A, Rabbani M, Sharifzadeh M, Bagheri N. Acute and chronic tramadol administration impair spatial memory in rat. Res Pharm Sci. 2016;11(1):49–57.
56. Lubman DI, Cheetham A, Yucel M. Cannabis and adolescent brain development. Pharmacol Ther. 2015;148:1–16.
57. Lehmann O, Bertrand F, Jeltsch H, Morer M, Lazarus C, Will B, et al. 5,7–DHT–induced hippocampal 5–HT depletion attenuates behavioural deficits produced by 192 IgG–saporin lesions of septal cholinergic neurons in the rat. Eur J Neurosci. 2002;15(12):1991–2006.
58. Majlessi N, Kadkhodaei M, Parviz M, Naghdi N. Serotonin depletion in rat hippocampus attenuates L–NAME–induced spatial learning deficits. Brain Res. 2003;963(2):244–51.
59. Adams W, Kusljic S, van den Buuse M. Serotonin depletion in the dorsal and ventral hippocampus: effects on locomotor hyperactivity, prepulse inhibition and learning and memory. Neuropharmacol. 2008;55(6):1048–55.
60. Normile HJ, Jenden DJ, Kuhn DM, Wolf WA, Altman HJ. Effects of combined serotonin depletion and lesions of the nucleus basalis magnocellularis
on acquisition of a complex spatial discrimination task in the rat. Brain Res. 1990;536(1):245–50.
61. Sarikaya S, Gulcin I. Radical scavenging and antioxidant capacity of serotonin. Curr Bioact Comp. 2013;9(2):143–52.
62. Jackson R, McNeil B, Taylor C, Holl G, Ruff D, Gwebu E. Effect of aged garlic extract on Caspase–3 activity, in vitro. Nutr Neurosci. 2002;5(4):287–90.
63. Schwerk C, Schulze–Osthoff K. Non–apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol. 2003;66(8):1453–8.
64. Ghoneim FM, Khalaf HA, Elsamanoudy AZ, Helaly AN. Effect of chronic usage of tramadol on motor cerebral cortex and testicular tissues of adult male albino rats and the effect of its withdrawal: Histological, immunohistochemical and biochemical study. Int J Clinical Exp Pathol. 2014;7(11):7323–41.
65. Maschke M, Fehlings T, Kastrup O, Wilhelm HW, Leonhardt G. Toxic leukoencephalopathy after intravenous consumption of heroin and cocaine with unexpected clinical recovery. J Neurol. 1999;246(9):850–1.
66. Iversen L. Cannabis and the brain. Brain. 2003;126(6):1252–70.
67. Abrams DI, Couey P, Shade SB, Kelly ME, Benowitz NL. Cannabinoid–opioid interaction in chronic pain. Clin Pharmacol Ther. 2011;90(6):844–51.
68. Bartsch AJ, Homola G, Biller A, Smith SM, Weijers HG, Wiesbeck GA, et al. Manifestations of early brain recovery associated with abstinence from alcoholism. Brain. 2007;130(1):36–47.

Cite this article as: Nafea OE, ElKhishin IA, Awad OA, Mohamed DA. A study of the neurotoxic effects of tramadol and cannabis in adolescent male albino rats. Int J Sci Rep 2016;2(7):143-54.