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Research Article

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

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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.[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.[27-29]

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:

$$\text{SP (ml)} = \frac{\text{Sucrose intake (ml)}}{\text{Sucrose intake (ml)} \times \text{water intake (ml)}} \times 100$$

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 (10×10×10 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]

In the Morris water maze protocol, prior to the conduct of the experiment, rats were trained in a standard Morris spatial navigation task in a water pool. The pool was virtually divided into four sections, named N, S, W and E (start positions). Starting from four separate, randomly assigned 'start positions', rats were trained to find an invisible platform placed at a fixed position. Trials continued until the animal found the platform or until 120 seconds had elapsed. If the animal did not find the platform within 120 seconds, then it was placed on the platform for 20 seconds and removed from the water tank. Rats were trained for 4 consecutive days, receiving 4 trials per day at 30–min intervals. Videos were recorded for each trial and escape latencies were measured using a stopwatch. At the end of each trial, each rat was dried, returned to its home–cage.[36]

On the fifth day, rats were randomly arranged to the study groups. Rats were re–tested for memory retention, by the water maze test, (Postnatal date: PND 60 and 90). The latency to find hidden platform was calculated for each group.[37]

Brain biochemical assessment

The brains were perfused with a phosphate buffered saline (PBS), containing 0.16 mg /mL heparin to remove any red blood cells and clots, then the brains immediately excised, dried, weighed. One cerebral cortex and hippocampus were rapidly dissected and stored at –80^{o}C for measurement of serotonin level. Identification of their anatomical positions was guided by Spijker.[38] Then the remaining tissue of brain was weighed and divided into 2 parts. One part was equally divided into 2 parts; each was freshly used for the determination of oxidative stress markers. The other part was kept in 10% formalin for the histopathological study.

N.B. Elven rats were randomly selected from each group during the first month of the experiment for behaviour testing.

Estimation of serotonin (5–HT) levels in the cerebral cortex and hippocampus by high–pressure liquid chromatography (HPLC) analysis

Frozen tissues of cerebral cortex and hippocampus were weighed and then sonically disrupted in 0.16 N perchloric acid ($HClO_4$). Removal of insoluble protein was done by centrifugation at 14,000 ×g for 5 min at 22 °C. The resulting supernatant was collected and diluted 1:4 in fluorescence dilution solution (0.01M HCl, 0.5mM EDTA, and 1mg/ml ascorbic acid).[39] HPLC columns are packed with very fine particles (stationary phase). The separation was done on Hypersil C18 (5 µm, 100 x 4.6 mm) columns, Surveyor, ThermoScientific Company, USA, with mobile phase as described later and the flow rate was 1.5 ml/min 25^{o}C.

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.[39]

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 eosin 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 u. 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 Labaratories– Cat.≠: 1475–1) using the avidin biotin peroxidase method.[40]

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.[41]

Optical density was calculated by the following formula:

Optical density = log (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.[42]

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).

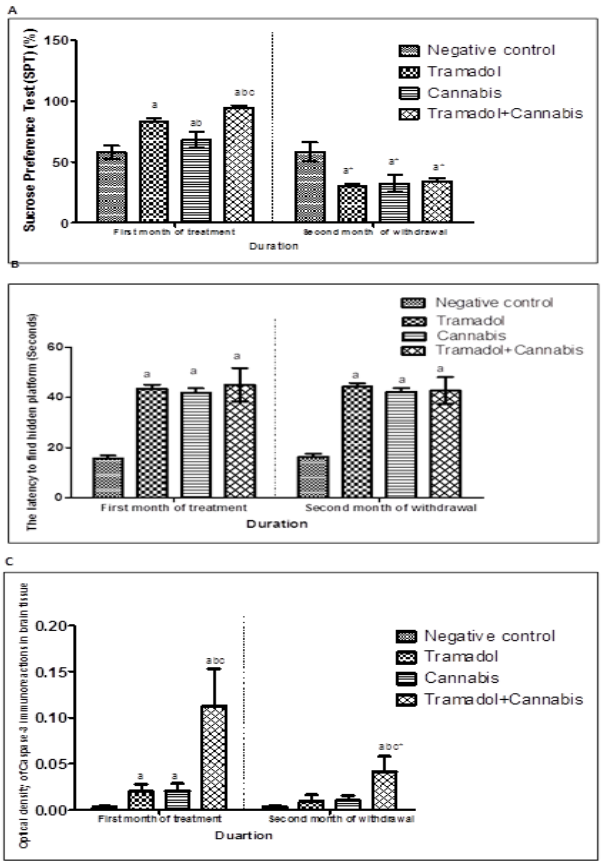

Figure 1: Shows effects of tramadol, cannabis and their combination on behavioral tests (SPT; sucrose preference test, MWM; Morris water maze), (A) sucrose preference and (B) latency to find a hidden platform and (C) the optical density of Caspase–3 immunoreactions in the brain tissue after the first month of treatment and the second month of withdrawal.

Values are expressed as mean±SD. Two way ANOVA test with Bonferroni post hoc test (significant if p ≤0.05), [a] significant vs negative control group, [b] significant vs TRM, [c] significant vs CANN of the same period (treatment or withdrawal). * Significant vs the same group after the treatment period compared to the withdrawal period (Bonferroni adjusted t–test).

Morris water maze test

Two–way ANOVA of the latency to find a hidden platform (Figure 3B) showed a non–significant main effect of duration on the latency to find a hidden platform ($F_{1,80}$ =0.03; p>0.05) but a significant overall effect of treatment on the latency to find hidden platform ($F_{3,80}$ =154.28; 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

Hematoxylin 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 eosinophilic 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[a]	0.48±0.11*
	CANN	18.66±3.37[a]	0.42±0.13*
	TRM+CANN	43.54±4.76[abc]	0.45±0.15*
Brain MDA (nmol/g. tissue)	**Negative control**	0.47±0.05	0.51±0.05
	TRM	18.38±3.64[a]	9.14±2.30[a]*
	CANN	15.48±2.30[ab]	7.16±1.36[a]*
	TRM+CANN	21.36±3.70[abc]	10.04±1.21[ac]*
Brain CAT (U/g)	**Negative control**	43.21±4.68	43.48±4.67
	TRM	19.65±3.97[a]	35.04±4.74 [a]*
	CANN	21.55±4.45[a]	37.05±4.02 [a]*
	TRM+CANN	15.51±2.93[abc]	34.85±6.29 [a]*

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

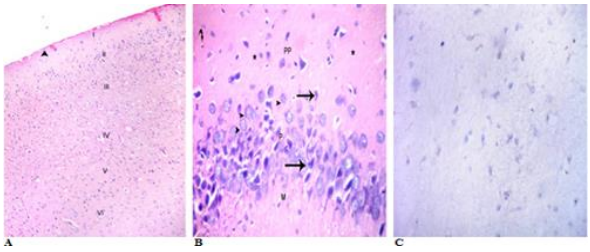

Figure 2: Shows a section of brain of negative control group. Panel (A) shows a delicate layer of pia matter (arrowhead) and the typical layered appearance of the cerebral cortex labelled I–VI as follows: I–Molecular layer; II–External granular layer; III–Pyramidal cell layer; IV–Internal granular layer; V–Ganglionic layer and VI–Multiform layer (H&E × 100). Panel (B) shows normal histo–architecture. 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).

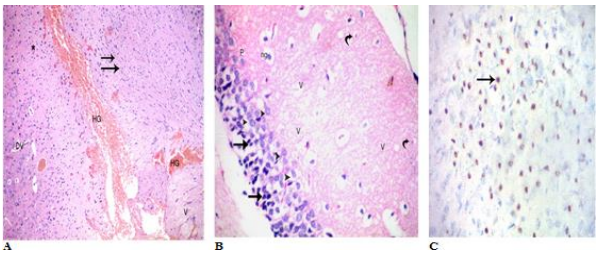

Figure 3: Shows a section of brain of tramadol–treated group. Panel (A) shows neuronal cell disorganization and hypercellularity, as well as increased apoptotic cells (arrows) and dilated blood vessel (DV). Note areas of severe haemorrhage (HG). Vacuolations of neuropil (V) and few red neurons (arrowhead) are evident. (H&E×100). 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 ×400). Panel (C) shows many neurons with strong positive reactions to the Caspase–3 antibody in their cytoplasm (Caspase immunostaining ×400).

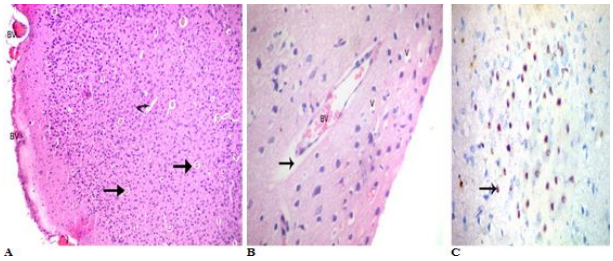

Figure 4: Shows a section of brain of cannabis–treated group. Panel (A) shows congested blood vessels (BV) in meninges. Irregular shrunken cells and vacuolated cytoplasm (arrows) are seen. The cells are surrounded by irregular wide spaces (curved arrows). (H&E × 100).Panel (B) shows congested blood vessels (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).

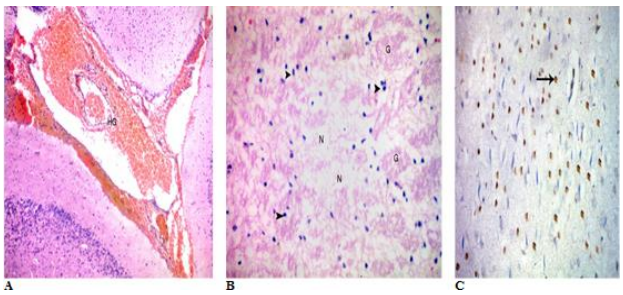

Figure 5: Shows a section of brain of tramadol+cannabis–treated group. Panel (A) shows areas of severe haemorrhage (HG) in the hippocampus area (H&E × 100). Panel (B) shows neuronal loss with degenerative changes (N) and gliosis (G). Most nuclei are apoptotic and destructed (arrowheads) (H&E × 400).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

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).

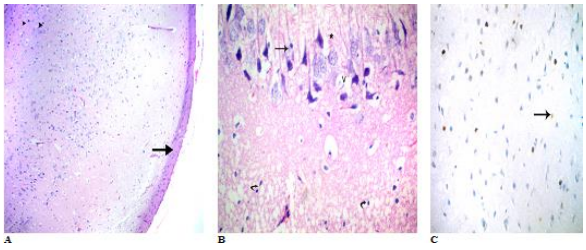

Figure 6: Shows a section of brain of tramadol–withdrawn group. Panel (A) shows thick meninges (arrow). Some nuclei are apoptotic (arrowheads) while other nuclei appear nearly normal (H&E × 100). Panel (B) shows decreased thickness of pyramidal layer (*). Vacuolations (V) in the pyramidal layer are seen. 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).

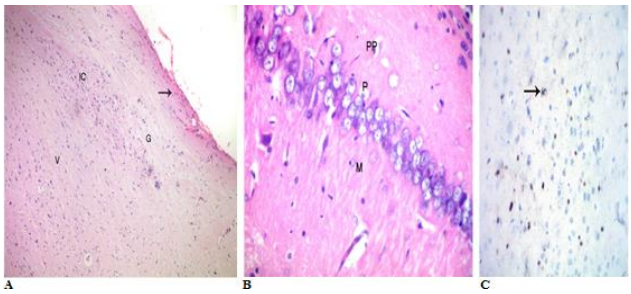

Figure 7: Shows a section of brain of cannabis–withdrawn group. Panel (A) shows inflammatory cellular infiltration (IC). Vacuolations (V) are still evident. An area of gliosis (G) is seen. Thick meninges (arrow) are noted (H&E × 100). Panel (B) shows nearly normal appearance of the hippocampus with its 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.

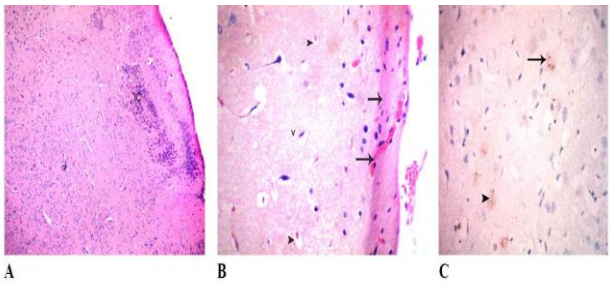

Figure 8: Shows a section of brain of tramadol+cannabis–withdrawn group. Panel (A) shows inflammatory cellular infiltrations (IC) (H&E × 100). Panel (B) shows thick congested oedematous meninges (arrows). Some red neurons (arrowheads) are seen. Areas of vacuolations (V) are still evident (H&E × 400). 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 ×400).

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 ($F_{3,80}$=91.85; p<0.0001) and duration ($F_{1,80}$ =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 serotoninergic transmission has been involved in the development of depression and related disorders.[45] 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.[60] 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.[61]

Surprisingly, Sarikaya and Gülçin proved that serotonin possess antioxidant and radical scavenging capacity by different in vitro methodologies.[62]

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.[63,64]

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.[65]

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.[66]

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.[67] 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.[22]

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.[68]

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.

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